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Latent TGF-β Binding Proteins:

Adhesive functions and matrix association of LTBP-2 and potential functions of LTBP-1 and LTBP-3 in mesothelioma

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Contents

List of original publications

This thesis is based on the following publications which are referred to in the text by their roman numerals:

I Vehviläinen, P., Hyytiäinen, M., And Keski-Oja, J.: Latent transforming growth factor-β-binding protein –2 is an adhesion molecule for melanoma cells. **J. Biol. Chem. 278,** 24705-24713, 2003

II Vehviläinen, P., Hyytiäinen, M., and Keski-Oja, J.: Association of latent TGF-beta binding protein LTBP-2 with the extracellular matrix is dependent on fibrillin-1. **J. Cell. Physiol. 221,** 586-593, 2009

III Tatti, O., Vehviläinen, P., Lehti, K., and Keski-Oja, J.: MT1-MMP releases latent TGF-β1 from endothelial cell extracellular matrix via proteolytic processing of LTBP-1. **Exp. Cell. Res. 314,** 2501-2514, 2008

IV Vehviläinen, P., Koli, K., Myllärniemi, M., Lindholm, P, Soini, Y., Salmenkivi, K., Kinnula, VL, and Keski-Oja J.: Latent TGF-β binding proteins (LTBPs)-1 and -3 differentially regulate TGF-β activity in malignant mesothelioma. Manuscript submitted.

Abbreviations

ADAM	a disintegrin-like and metalloproteinase
ADAMTS	ADAM with thrombospondin type I motifs
ALK	Activin receptor –like kinase
BM	basement membrane
BMP	bone morphogenic protein
BSA	bovine serum albumin
COL	collagenous domain
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FN	fibronectin
GAG	glycosaminoglycan
HS	heparan sulphate
HSPG	heparan sulphate proteoglycan
HUVEC	human umbilical vein endothelial cells
Ig	immunoglobulin
kDa	kilodalton
LAP	latency associated peptide
LL-TGF-β	large latent TGF-β complex
LTBP	latent TGF-β binding protein
MAGP	microfibril associated glycoprotein
MAPK	mitogen activated protein kinase
MEM	minimum essential medium
MM	malignant mesothelioma
MMP	matrix metalloproteinase
mRNA	messenger RNA
MT-MMP	membrane type MMP
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PFA	paraformaldehyde
PG	proteoglycan
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RT-PCR	reverse transcriptase polymerase chain reaction
shRNA	short hairpin RNA
SL-TGF-β	small latent TGF-β complex

NC	noncollagenous domain
TGF-β	transforming growth factor -β
TIMP	tissue inhibitor of matrix metalloproteinase
uPA	urokinase type plasminogen activator
uPAR	urokinase type plasminogen activator receptor
wt	wild type

Abstract

Latent transforming growth factor β (TGF-β) binding proteins (LTBPs) -1, -3 and -4 are ECM components whose major function is to augment the secretion and matrix targeting of TGF-β, a multipotent cytokine. LTBP-2 does not bind small latent TGF-β but has suggested functions as a structural protein in ECM microfibrils. In the current work we focused on analyzing possible adhesive functions of LTBP-2 as well as on characterizing the kinetics and regulation of LTBP-2 secretion and ECM deposition. We also explored the role of TGF-β binding LTBPs in endothelial cells activated to mimic angiogenesis as well as in malignant mesothelioma.

We analyzed numerous cell lines for their ability to adhere to purified recombinant LTBP-2. We found that, unlike most adherent cells, several melanoma cell lines adhered to LTBP-2 in an efficient and concentration dependent manner. Characterization of the most strongly adhering cell line with function blocking anti-integrin antibodies revealed that the adhesion was mediated by $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. In addition, soluble heparin inhibited the melanoma cell adhesion suggesting a role for heparan sulphate proteoglycans. Unexpectedly, the adhesion was not mediated by the N-terminal RGD sequence but was located to an adjacent proline-rich region. LTBP-2 was also identified as a haptotactic substrate for melanoma cell migration.

The deposition of latent TGF-β binding LTBPs to the ECM exhibit distinct distribution profiles. We used cultured human embryonic lung fibroblasts to analyze the temporal and spatial association of LTBP-2 into ECM. By immunofluorescence we found that LTBP-2 was efficiently assembled to the ECM only in confluent cultures following the deposition of fibronectin (FN) and fibrillin-1. Analysis by metabolic labeling and immunoprecipitation showed that LTBP-2 was rapidly secreted and subsequently associated with the ECM in confluent cultures whereas in early, subconfluent cultures it remained primarily in soluble form after secretion. LTBP-2 colocalized transiently with FN and failed to assemble to the ECM of FN deficient mouse fibroblasts. Partial colocalization of LTBP-2 and fibrillin-1 was also observed in the ECM of fibroblasts, MG-63 osteosarcoma cells and human vascular endothelial cells. Silencing of fibrillin-1 expression by lentiviral shRNAs profoundly disrupted the deposition of LTBP-2 indicating that the matrix association of LTBP-2 depends on a pre-formed fibrillin-1 network.

Considering the established role of TGF-β as a regulator of angiogenesis we induced morphological activation of endothelial cells by phorbol 12-myristate 13-acetate (PMA) and followed the fate of LTBP-1 in the endothelial ECM. This resulted in profound proteolytic processing of LTBP-1 and release of latent TGF-β complexes from the ECM. The processing was coupled with increased activation of MT-MMPs and specific upregulation of MT1-MMP. The major role of MT1-MMP in the proteolysis of LTBP-1 was confirmed by suppressing the expression with lentivirally induced short-hairpin RNAs as well as by the inhibition with tissue inhibitors of metalloproteinases (TIMPs) -2 and -3.

Malignant mesothelioma (MM) is an aggressive tumor of the pleura with poor prognosis. TGF-β can promote mesothelioma tumorigenesis through multiple mechanisms. We investigated the expression patterns of different TGF-β and LTBP isoforms in MM biopsies and in two established MM cell lines. TGF-β activity was analyzed in a panel of MM tumors by immunohistochemical staining of phosphorylated Smad-2 (P-Smad2). The tumor cells were strongly positive for P-Smad2 whereas LTBP-1 immunoreactivity was abundant in the stroma, and there was a negative correlation between LTBP-1 and P-Smad2 staining. In addition, the high P-Smad2 immunoreactivity correlated with shorter survival of patients. TGF-β1 was the most highly expressed isoform in both normal human pleura and MM tissue as assessed by quantitative RT-PCR. LTBP-1 and LTBP-3 were both abundantly expressed. mRNA analysis of cell lines showed that LTBP-1 was the predominant isoform in MM cells whereas the expression of LTBP-3 was markedly high in non-malignant control cells. Suppression of LTBP-3 expression by siRNAs resulted in increased TGF-β activity in MM cell lines accompanied by decreased proliferation of MM cells. Our results suggest that decreased expression of LTBP-3 in MM could alter the targeting of TGF-β to the ECM and lead to its increased activation.

The current work emphasizes the coordinated process of the assembly and appropriate targeting of LTBPs with distinct adhesive or cytokine harboring properties into the ECM. The hierarchical assembly may have implications in the modulation of signaling events during morphogenesis and tissue remodeling.

1 Review of the literature

1.1 Extracellular matrix

Extracellular matrix (ECM) is composed of diverse components which form complex networks where cells are embedded to form tissues (Aumailley and Gayraud 1998; Nelson and Bissell 2006). ECM provides the cells with structural support for adhesion and migration and defines the organ specific architecture of tissues. It also acts as storage for growth factors and signaling molecules affecting their activity and availability (Taipale and Keski-Oja 1997; Streuli 1999). The interactions between cells and ECM regulate many fundamental processes during the normal development as well as in disease (Nelson and Bissell 2006). Despite the diversity of ECM molecules most of them share similar structural domains. Thus the function of the ECM is not solely dependent on single molecules but on the multiprotein polymeric assemblies.

1.1.1 Structure

ECM structures can be classified into the interstitial matrix, which accounts for the connective tissue of different types, and the basement membrane, which lines the endothelium and epithelium separating them from the underlying stroma.

1.1.1.1 Interstitial matrix

Interstitial matrix is a highly hydrated fibrillar network that fills the space between the cells and determines the physical properties of the given tissue. It can comprise greater mass of an organ than the cells that produce its components. The main constituents of the interstitial ECM are proteoglycans (PGs) and fibrillar proteins, such as collagen, fibronectin (FN) and elastin, and they are primarily produced by fibroblasts. The aqueous environment of polysaccharide gels allows rapid diffusion of small molecules such as salts, nutrients and hormones whereas the protein components provide support and adhesion sites for cells. The relative amounts of matrix components vary and give rise to a diversity of tissues with distinct functional properties. FN is found in most ECMs but it is especially abundant during embryogenesis and in the provisional matrix of injured tissue orchestrating the assembly of many other ECM proteins (Magnusson and Mosher 1998). It is also an important adhesion molecule for various cell types. Collagens are the most abundant structural elements in adult tissues providing them tensile strength. Collagen fibrils can be arranged in different three dimensional arrays: they appear as parallel bundles in tendons and ligaments, orthogonal lattices in the cornea and concentric weaves in bone (Hulmes 2002; Canty and Kadler 2005). Elastic fibers are able to recoil after

transient stretching and they are found in tissues such as skin, blood vessels and lung where strength and elasticity are needed (Mecham 1991).

1.1.1.2 Basement membrane

Basement membranes (BMs) are specialized forms of ECM with thin, sheet-like structure. They are closely associated with epithelial or endothelial cells, muscle fibers, adipocytes and peripheral nerves. BMs provide cells with structural support and regulate cell polarity. They separate tissues into different compartments and act as molecular filters. In addition, BMs regulate cell behavior through interactions between cell surface receptors and ECM molecules (Kalluri 2003). The major constituent of BMs is type IV collagen, and other main elements include laminin, heparan sulphate PGs (HSPGs) and nidogen/entactin. There are also small amounts of other components including fibulins, type XV and XVIII collagens and SPARC/osteopontin. The molecular composition of BMs is not uniform throughout the body but displays organ-specific differences. For example, type IV collagen, laminin and PGs exist in several isoforms whose expression patterns vary in different organs.

Laminin and type IV collagen are capable of initiating the self-assembly of BM molecules into sheet-like structures. Laminin is a centerpiece of the structure deposited first on the cell surface while the subsequent assembly of type IV collagen provides highly crosslinked scaffold for the BM (Timpl 1996; Kalluri 2003). Collagen IV is a member of collagen family of trimeric protein consisting of three α-chains. There are six genetically different α-chains of type IV collagen and they form three triple helical protomers with different chain compositions (Hudson et al. 2003). The expression patterns of the protomers differ depending on the developmental stage and tissue. Laminins, which are also trimers, consist of α, β and γ chains which give rise to at least 12 different laminin isoforms (Timpl 1996). They form networks of mainly noncovalent nature. The formation of the connected laminin and collagen IV networks is mediated by nidogen and promoted by complex interactions between various BM molecules and also via interactions with cell surface receptors (Kalluri 2003).

1.1.2. Collagens

1.1.2.1 Collagens are a large family of ECM proteins

Collagens are the most abundant proteins in human body. They are homo- or heterodimers of three polypeptides called α-chains. Total of 42 different α-chains exist in vertebrates which can form at least 28 types of collagen molecules (Myllyharju and Kivirikko 2004; Kadler et al. 2007). The α-chains typically have repeating Gly-X-Y sequences which form so called collagenous domains (COL) where the glycine residue is essential for the coiled-

coil structure. X and Y positions can hold any other amino acid but are often occupied by proline and 4-hydroxyproline the latter providing stability for the triple helix. In addition, all collagens have also non-collagenous (NC) domains which can be even more abundant in certain collagens than the COL domains. The superfamily of collagens is thus very heterogeneous and it is further dived into several subfamilies based on their ability to form different supramolecular assemblies or other properties. The collagens are designated with upper case and the subfamilies with lower case Roman numerals, respectively.

Fibril-forming collagens (i) are important in providing tissues with tensile strength. They are a rather homogeneous group that includes collagen types I, II, III, V, XI, XXIV and XXVII of which type I collagen is the most abundant and a classical example of a fibrillar collagen (Myllyharju and Kivirikko 2004; Kadler et al. 2007). The group of network forming collagens (ii) consists of collagen types IV, VIII and X of which type IV is an important component of BMs functioning as a molecular filter. Collagen type VIII is found in Descemet's membranes and vascular subendothelial matrices and type X in hypertrophic cartilage. Collagens of types IX, XII, XIV, XVI and XIX-XXII comprise a family of fibril-associated collagens (iii) with interrupted triple helices (FACITs) because of their structure and presence on the surface of collagen fibrils. The beaded-filament-forming collagens (iv) are found in microfibrils and include collagen types VI, XXVI and XXVIII. Type VII collagen is present in dermal-epidermal junction attaching BM and epithelia to the stroma and is thus called anchoring fibril type of collagen (v). Transmembrane collagens (XIII, XVII, XXIII and XXV)(vi) have short cytosolic domains and large extracellular domains which have adhesive functions. Collagens XV and XIII (vii) are sources of biologically active fragments endostatin and restin, respectively, which inhibit endothelial cell migration and angiogenesis.

Some collagen types such as I, III, V, VI, XII and XIV are found in many types of ECM whereas the distribution of certain collagens is very restricted (Myllyharju and Kivirikko 2004; Canty and Kadler 2005). In addition to subfamilies mentioned above, there are several COL domains containing proteins that are not classified as collagens, such as a subcomponent of C1q of complement, many collectins and some ficolins and macrophage receptors, all of which function in immune system. Further, adiponectin, acetylcholinesterase, ectodysplasin and two EMILINs also have COL domains.

The importance of collagen types of ECM proteins is emphasized by the fact that they are evolutionarily conserved: most invertebrates have examples of fibrillar and BM collagens (Kadler et al. 1996; Huxley-Jones et al. 2007). Moreover, mutations in the genes encoding collagens cause a variety of human diseases that include osteogenesis imperfecta, chondrodysplasias, various types of Ehlers-Danlos syndrome, arterial and intracranial aneurysms, epidermolysis bullosa, the renal disease known as Alport syndrome and others (Myllyharju and Kivirikko 2004).

1.1.2.2 Collagen assembly

Collagens are synthesized as soluble precursors, procollagens, which undergo extensive posttranslational modifications assisted by a number of molecular chaperones and

enzymes (Hulmes 2002; Canty and Kadler 2005). The C-terminal propeptide directs the association between the three α-chains during assembly in the endoplasmic reticulum (ER). The C-propeptide also confers to the high solubility of the procollagen and thus prevents too early fibril formation. The N-propeptide affects the diameter of the forming fibrils. The N- and C-terminal propeptides are cleaved by specific proteinases during or following the secretion of the procollagen molecule. Covalent crosslinks are finally formed within and between triple-helical collagen molecules in the growing fibril.

Purified collagen molecules can self-assemble into fibers in cell-free systems *in vitro* (Kadler et al. 1996). However, the *in vivo* situation is much more complex as collagens interact with other types of collagens and, in addition, with a number of other proteins (Kadler et al. 2008). A growing body of evidence suggests that FN and integrins are essential for collagen fibril formation (Velling et al. 2002; Li et al. 2003). Inhibition of either FN assembly or the function of certain integrins inhibits collagen fiber assembly. However, the mechanism how FN and integrins catalyze the fibrillogenesis of collagen is not known. Some minor collagens have been suggested to function as nucleators of the process. For example, type V collagen, which is codistributed with type I collagen, is essential for the assembly of collagen I fibrils *in vivo* (Wenstrup et al. 2004). Mouse embryos lacking collagen V die of cardiovascular failure likely due to the lack of collagen fibrils in the mesenchyme. Collagen XI is structurally homologous to collagen V and associates with collagens II and IX in cartilage where it is suggested to function as a nucleator of the assembly of thin fibers (Kadler et al. 2008). In addition, several other molecules, such as decorin, fibromodulin, lumican, perlecan, biglycan and tenascin-C, have been shown to affect the formation of collagen fibers (Canty and Kadler 2005; Kadler et al. 2008).

1.1.3 Fibronectin

1.1.3.1 Fibronectin is a large glycoprotein of the provisional matrix and blood plasma

Fibronectin (FN) is a large 250 kDa glycoprotein that was first identified from blood plasma and on the surface of fibroblasts (Ruoslahti and Vaheri 1974). It is an abundant fibrillar component of provisional matrix but it is also found in most interstitial matrices and BM (Magnusson and Mosher 1998). Soluble plasma FN is synthesized by hepatocytes in the liver whereas in tissues FN is secreted locally by a number of cell types. The fundamental role of FN in the ECM was observed by analysis of the knock-out mouse which dies at day 8.5 of embryonic development (George et al. 1993). The embryos have defects in vascular development, neural tubes and mesodermally derived tissues.

FN is synthesized as a soluble disulphide-bonded dimer. Both subunits consist of type I, II and III repeats and a variable sequence (Magnusson and Mosher 1998). Two of the type III repeats are subject to alternative splicing and are absent in plasma FN. The variable region can also be spliced out. Different regions confer different properties of which the most important is the Arg-Gly-Asp (RGD) sequence in the III10 repeat which

mediates the integrin binding to FN (Pierschbacher and Ruoslahti 1984; Pytela et al. 1985). In addition to integrin receptors, FN harbors binding sites for several other molecules including fibrin, collagen, heparin and PGs (Ruoslahti et al. 1982).

1.1.3.2 Assembly of fibronectin

FN matrix assembly is a stepwise cell-mediated process (Magnusson and Mosher 1998; Wierzbicka-Patynowski and Schwarzbauer 2003; Mao and Schwarzbauer 2005). First the secreted soluble FN binds to cell surface primarily via $\alpha_5\beta_1$ integrin although other integrins can substitute for it under some conditions. As FN is a dimeric ligand this engagement induces integrin clustering and local accumulation of FN which, in turn, promotes FN organization into short fibrils. The interactions between the cytoplasmic domains of integrins and the actin cytoskeleton are essential for FN assembly. Several signaling molecules are recruited to these sites and lead to generation of contractile forces that presumably stretch the FN molecules exposing intermolecular interaction sites and thus promoting fibril formation. As the fibrils grow soluble FN is also recruited into the fibrils through FN-FN interactions in an integrin-independent manner. The extension of FN requires disruption of interdomain interactions but the mechanism is poorly understood. In addition to integrins, there are other cell-surface or transmembrane molecules that affect FN assembly: syndecan-4 and transglutaminase promote FN assembly via co-operation with the $\alpha_5\beta_1$ integrin (Wierzbicka-Patynowski and Schwarzbauer 2003; Akimov and Belkin 2001). The urokinase receptor for plasminogen activator (uPAR) modulates FN assembly by regulating the activation of the $\alpha_5\beta_1$ integrin (Monaghan et al. 2004).

Even though the mature FN matrix is relatively stable it is not a static structure. Cell movements and detachment affect the length and shape of FN fibrils. Furthermore, continuous polymerization of FN is critical for the maintenance of cell surface FN fibrils and for the deposition of other ECM molecules such as type I collagen and thrombospondin (Sottile and Hocking 2002).

1.1.4 Elastic fibers

1.1.4.1 Elastic fibers are assemblies of elastin and microfibrils

Elastin is a major component of elastic fibers that are found virtually in all mammalian connective tissues (Mecham 1991; Kielty et al. 2002). The unique elastomeric properties of elastin provide tissues with reversible deformability which is of special importance in arterial vessels, lungs and skin. Elastin fibers are rubber-like polymers capable of being rapidly stretched to two to three times their resting length. After release of tension they are passively recoiled to the original size. For example, this feature allows the alveolar

expansion and recoil during breathing. The architecture of the elastic fibers varies in different tissues reflecting tissue specific functions.

Elastic fibers consist of two morphologically distinct components: an inner core of insoluble amorphous appearing elastin and an outer microfibrillar shell (Mecham 1991; Kielty et al. 2002). Elastin is synthesized as a soluble ~70 kDa precursor, tropoelastin, which, in contrast to collagens, undergoes only minor posttranslational modifications. The polymeric, mature elastin is one of the most insoluble proteins in the body and very resistant to the extreme conditions used to isolate other ECM proteins. It is also very stable under physiological conditions the halftime approaching the lifetime of an organism. Mature elastin has a high content of non-polar amino acids and is thus extremely hydrophobic.

Microfibrils are 10-12 nm fibrillar assemblies mainly composed of fibrillins (Kielty et al. 2002). They act as structural scaffold for tropoelastin deposition but are also abundant in connective tissues devoid of elastin. There are three different fibrillins, 1-3 (Sakai et al. 1986; Zhang et al. 1994; Nagase et al. 2001), and they are large (~350 kDa) glycoproteins with long stacks of EGF-like repeats and several cysteine-rich domains typical for LTBP-fibrillin superfamily of proteins (see also 1.3.2 Fibrillins). Fibrillin-2 is expressed during early development before the appearance of fibrillin-1. Later, fibrillin-1 is more abundant and is expressed in various tissues.

In addition to elastin and fibrillins, there are several other proteins associated with elastic fibers. Microfibril-associated glycoprotein (MAGP) -1 is widely distributed associating with most microfibrils and is suggested to be an integral component of microfibrils (Gibson et al. 1989; Trask et al. 2000a). The tissue localization of the related MAGP-2 is more restricted (Gibson et al. 1998). Latent TGF-β binding proteins (see also section 1.3.2 LTBPs and (Hyytiäinen et al. 2004)) belong to the same superfamily with fibrillins, and LTBPs -1, -2 and -4 interact with one or more fibrillin (Isogai et al. 2003; Hirani et al. 2007; Ono et al. 2009). Fibulins -1, -2, -4 and -5, in turn, bind tropoelastin (Timpl et al. 2003; McLaughlin et al. 2006). EMILINs -1 and -2 localize to elastin fibers and at least EMILIN-1 binds to elastin (Doliana et al. 1999; Doliana et al. 2001). Several PGs, such as decorin, biglycan and versican have been detected in elastic fibers and may contribute to their integration into surrounding ECM (Kielty et al. 2002).

1.1.4.2 Assembly of microfibrils and elastic fibers

The microfibrillar template is formed first and determines the localization and morphology of the elastin fibers (Mecham 1991; Kielty et al. 2002). Microfibrils appear as beads-on-string-like structures when visualized by various microscopic techniques. Most of current knowledge on the assembly of microfibrils is limited to fibrillin-1. The assembly of the fibrillin-1 fibrils is at least partially mediated by cells and starts in the secretory pathway by C-terminal processing of profibrillin by a furin convertase (Ritty et al. 1999; Raghunath et al. 1999). Removal of the C-terminus is needed for the ECM deposition. This is followed by rapid formation of N-terminal intermolecular disulphide bonds which confer to the fiber insolubility (Trask et al. 1999; Reinhardt et al. 2000). The cross-linking

takes place either inside the cell or shortly after secretion. The organization of fibrillin-1 into microfibrils requires the multimerization of the C-terminus into the bead-like structures which then allows the self-interaction of the N- and C-termini (Hubmacher et al. 2008).

After the formation of the microfibril scaffold the tropoelastin is thought to be deposited onto it (Mecham 1991). This model is supported by the observed ability of tropoelastin to bind fibrillin-1 and -2 (Trask et al. 2000b). Next the tropoelastin undergoes coacervation (self-aggregation) and becomes crosslinked by lysyl oxidase.

The surrounding ECM and many interacting proteins may affect the assembly of microfibrils and elastic fibers as well as their commitment to distinct ECM sites. For example FN assembly is required for the deposition of fibrillin-1 (Kinsey et al. 2008). Fibrillins -1 and -2 contain heparin binding sites that are important for matrix association (Ritty et al. 2003), and heparin can compete with tropoelastin for binding to fibrillin-1 (Cain et al. 2005). Furthermore, fibulins-4 and fibulin-5 are critical for the maturation of elastic fibers via interactions with tropoelastin and fibrillin-1 (McLaughlin et al. 2006; Nakamura et al. 2002; Freeman et al. 2005; Zheng et al. 2007; Choudhury et al. 2009). EMILIN-1 deficiency disrupts the elastin core without affecting microfibrils (Zanetti et al. 2004). MAGP-1 is directly involved in elastin fiber assembly and MAGP-2 is able to stimulate it (Brown-Augsburger et al. 1996; Lemaire et al. 2007).

1.1.5 Proteoglycans

Proteoglycans (PGs) are a diverse group of molecules which are classified after their major constituents, sulphated sugar chains, also called glycosaminoglycans (GAGs) (Ruoslahti 1988; Hardingham and Fosang 1992). GAGs, which often account for more than 80% of the mass of a PG, are attached to the smaller protein core. The sugar side chains vary both in structure and in size, and also the core proteins are extremely diverse depending on the cell type they are produced by. PGs are highly hydrophilic and bind large amount water occupying a lot of space thus providing tissues with amorphous ground substance. They also interact with various other molecules affecting many cellular functions such as ECM assembly, morphogenesis, cell growth and differentiation, tumorigenesis and modulation of growth factor functions. PGs can be divided into three groups based on the structure of the GAGs they carry: i) chondroitin/dermatan sulfate PGs (CSPGs/DSPGs), ii) heparan sulfate PGs (HSPG) and iii) keratan sulfate PGs (KSPGs) (Gesslbauer et al. 2007). All three types of GAGs are strongly negatively charged, and the interactions between PGs and other molecules are ionic in nature. The core proteins of PGs include perlecan, agrin and bamacan present in BM; versican, decorin, and biglycan mainly found in skin; and aggrecan, neurocan and brevican of which aggrecan is abundant in cartilage and neurocan is found in brain (Gesslbauer et al. 2007; Iozzo 1998).

1.1.6 Remodeling of ECM

Cells need to respond to environmental stimuli for example during embryogenesis, wound healing as well as under pathological conditions (Streuli 1999). The adaptation leads to temporal changes in the composition of the ECM. Moreover, the proteolysis of ECM molecules exposes previously unavailable sites for cell surface receptors or releases growth factors and other biologically active molecules which affect cell behavior. The balance between ECM synthesis and degradation is important and is shifted towards accumulation of ECM proteins during fibrosis and scarring. A shift towards excessive remodeling is seen in arthritis and tumor development (Tlsty and Coussens 2006). During early development the remodeling events are required for the differentiation of diverse cell types derived from primitive epithelium (Streuli 1999). In the adult, angiogenesis and wound healing as well as uterine and mammary gland changes during the menstrual cycle and pregnancy are dependent on ECM remodeling. There are numerous enzymes involved in these processes including families of serine proteases and metalloproteases.

Enzymes of the plasmin/uPA system are serine proteases which are especially important during wound healing. Thrombin cleaves fibrinogen to form fibrin which forms the fibrin clot after wounding (Toriseva and Kähäri 2009). Plasmin has a key role in dissolving the fibrin clot to facilitate the migration of the keratinocytes in the healing wound. It is abundantly present in blood plasma as a precursor, plasminogen, which is activated by plasminogen activators (uPA/tPA), which, in turn, are inhibited by plasminogen activator inhibitors (PAI-1 and -2) (Qiu et al. 2007; Andreasen et al. 2000). uPA can be specifically targeted to the cell membrane via interaction with its receptor, uPAR, to regulate cell migration and invasion. Plasmin and uPA are involved in digesting FN and laminin, and regulating the activity of coagulation factors. They can also activate several MMPs and growth factors such as hepatocyte growth factor and TGF-β.

MMPs are a family of 23 highly homologous, mainly secreted or plasma membrane-associated proteinases that require a zinc ion for their catalytic activity (Nagase et al. 2006). Together they can digest virtually all ECM molecules and, in addition, many latent growth factors and cytokines, cell surface receptors, adhesion proteins and other pro-proteinases and proteinase inhibitors. For example, MMPs contribute to vascular remodeling by degrading the matrix proteins as well as processing vascular endothelial growth factor (VEGF), an important initiator of angiogenesis (Page-McCaw et al. 2007). MMPs are divided into subgroups based on their substrate preferences and domain organization: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs) and a group of other MMPs (Nagase et al. 2006). All MMPs have a prodomain that maintains the enzyme in inactive form, and a catalytic domain which interacts with the prodomain through the zinc ion. The hemopexin domain participates in determining substrate specificity and mediates interactions with MMP inhibitors or between two MT-MMPs. Through complex formation of MMPs with ECM substrates and their cell surface receptors the proteolytic activity can be restricted for example to the leading edge of the migrating cell. The activity of MMPs is regulated by α2-macroglobulin and related proteins present in blood plasma and tissue inhibitors of metalloproteinases (TIMPs), which display different specificities for individual MMPs.

Roles for several MMPs and their inhibitors have been implicated in different phases of wound healing (Toriseva and Kähäri 2009). MMPs -2, -9, -13 and -14 (MT1-MMP) are involved in bone development, fracture healing and bone remodeling and MMPs -2 and -3 are important for the morphogenesis of the mammary gland (Page-McCaw et al. 2007). Interestingly, among the numerous *mmp* knockout mice animals with disrupted MT1-MMP exhibit the only lethal phenotype. They suffer from skeletal remodeling defects, arthritis, osteopenia and angiogenic defects (Holmbeck et al. 1999; Zhou et al. 2000). The condition leads to death at 3-12 weeks.

A disintegrin and metalloproteinase family (ADAMs) and ADAM with thrombospondin motifs family (ADAMTSs) resemble MMPs as they also have zinc binding motif and are inhibited by TIMPs (Huovila et al. 2005). ADAMs generally exist as membrane bound and in a few cases as soluble forms and shed the extracellular domains of several membrane-bound molecules including growth factors, cytokines, adhesion proteins and receptors. In addition, they can also cleave and remodel components of the ECM. The disintegrin motifs of ADAMs mediate their interactions with several integrin receptors. ADAMTS have thrombospondin-like modules replacing the transmembrane and cytoplasmic domains and are thus soluble proteases (Porter et al. 2005). In contrast to both MMPs and ADAMs, some of the ADAMTSs associate with the ECM. ADAMTSs are involved in blood coagulation homeostasis, regulation of angiogenesis and processing of collagens. Their substrates include von Willebrand factor, aggrecan, versican and pro-collagens I, II and III.

1.1.7 Role of ECM in cancer

Uncontrolled and invasive growth typical for cancer does not solely rely on mutations in oncogenes and/or tumor suppressor genes (Tlsty and Coussens 2006; Bhowmick et al. 2004; Sund and Kalluri 2009). Instead, the reciprocal interactions between cancer cells, stromal cells as well as soluble and insoluble components of the surrounding ECM are essential for invasive growth. Fibroblasts are the predominant cells in the stroma and regulate the composition of the ECM in an organ-specific manner. In tumors they display unique and dynamically changing phenotypes and are usually referred as cancer (or carcinoma) -associated fibroblasts (CAFs) or myofibroblasts. Acquired expression of α-smooth muscle actin, vimentin, tenascin and desmin is a typical feature of these cells. Genetically altered neoplastic cells can induce the normal fibroblasts to become CAFs or myofibroblasts. Vice versa, inherited or acquired mutations in stromal fibroblasts may precede the neoplastic transformation of epithelial cells.

The architecture of the tumor-associated stroma is markedly different from the pre-existing stroma of healthy tissue. While the turnover of collagens is slow under normal conditions the metabolism of type I collagen is increased in neoplastic tissues and the newly synthesized collagen fibers are disorganized (Tlsty and Coussens 2006). This may promote angiogenesis necessary for tumor development as small blood vessels and capillaries are often abundant in tumor stroma and, moreover, high vasculature in the primary tumor indicates poor prognosis (Carmeliet and Jain 2000). The importance of

microenvironment permissive for tumor growth is emphasized by existence of *in situ* cancers which never invade (Folkman and Kalluri 2004). The dormancy of such tumors is thought to be due to the angiogenic defense of the host which includes factors such as thrombospondin, tumstatin and endostatin. In addition to regulation of angiogenesis, the altered structure of the stroma may also affect the availability of various mitogenic growth factors and thus promote tumor growth. In advanced cancer the establishment of metastases is also dependent on suitable microenvironment (Sund and Kalluri 2009).

Carcinoma, a malignant tumor arising from the epithelium, is the most common type of human cancer. Polarized three-dimensional structure is important for epithelial cells and their impaired interactions with the ECM may lead to the development of carcinoma (Bhowmick et al. 2004). Normal tissue architecture is thus an efficient inhibitor of tumorigenesis. Normal fibroblasts may prevent epithelium from becoming tumorigenic, whereas the accumulation of myofibroblasts leads to epithelial hyperproliferation and resistance to apoptosis (De Wever et al. 2008). Cancer cells are able to recruit myofibroblasts and induce their differentiation, proliferation and production of proinvasive signals. The soluble mediators secreted by myofibroblasts or CAFs include various cytokines, such as TGF-β and VEGF, and matrix remodeling enzymes.

Cancer can be described as a wound that does not heal. In wounded or injured tissue the damaged cells are cleared via induction of cell death pathways while cell proliferation and matrix production are enhanced to regenerate the tissue or close the wound (Tlsty and Coussens 2006). In cancer, the proliferation is sustained and the malignant cells have acquired the ability to escape apoptosis. Infiltration of inflammatory cells, namely macrophages, is an important phenomenon during wound healing. Macrophages are also present in tumor stroma where they may influence the ECM remodeling and facilitate invasion. Accordingly, chronic inflammation predisposes to various cancers. On the other hand, recruitment of adaptive inflammatory cells, e.g. B and T lymphocytes, may also restrict tumor growth.

MMPs can regulate tumorigenesis in various ways (Folgueras et al. 2004). Their expression or activity is elevated in most cancers and correlates with poor prognosis. As MMPs are able to degrade virtually all ECM components they clear the path for cancer cell invasion into surrounding stroma and into blood vessels. In addition, MMPs can modulate tumor angiogenesis, the immune functions and apoptotic processes by activating or releasing growth factors from the ECM reservoir or from plasma membrane association. However, MMPs can also release anti-angiogenic/anti-tumorigenic ECM fragments such as angiostatin, endostatin (fragment of collagen XVIII), restin (fragment of collagen XV) and fragments of collagen IV, arrestin, canstatin and tumstatin. Thus, the role of the proteolytic system in tumorigenesis is very complex.

1.2 Cell adhesion

The attachment of cells to each other and to the surrounding ECM is crucial for the formation and maintenance of tissue architecture. These interactions fundamentally regulate cell functions and phenotypes. There are variety of adhesion mechanisms utilized

by cells but often the processes and molecular components are similar from the morphogenesis throughout the life of an organism (Gumbiner 1996). The adhesive units consist of many types of proteins: ECM proteins, cell adhesion molecules/receptors, and the cytoplasmic plaque/peripheral membrane proteins. The cell adhesion receptors are often transmembrane proteins that mediate cell-cell or cell-ECM adhesion and include members of cadherin, immunoglobulin, selectin, integrin and PG superfamilies. On the cytoplasmic site of adhesive structures there are a number of molecules which participate in signal transduction or organization of the cytoskeleton (Zamir and Geiger 2001).

1.2.1 Cell-Cell contacts

Stable connections between the cells are required for the proper functions of tissues and organs. The most widespread types of adhesion contacts include adherens junctions, desmosomes and tight junctions (Gumbiner 1996).

Adheres junctions are formed by homophilic interactions between molecules of cadherin family (Gumbiner 1996). They are transmembrane proteins and highly expressed in various solid tissues. Widely studied example is E-cadherin which mediates stable adhesion between epithelial cells. Loss of its functions can not be compensated by other adhesion mechanisms and is associated with increased invasiveness of cells in culture and certain tumors. Adhesive function of cadherins requires interactions with cytoplasmic plaque proteins such as α- and β-catenins, and actin cytoskeleton. The adherens junctions between epithelial cells and associated actin filament bundles are analogous to focal adhesions at sites of integrin-ECM contacts and may provide resistance to contractile forces for example in the epithelium of digestive tract.

Desmosomes are sites of strong adhesion and anchored to intermediate filaments (Yin and Green 2004). They consist of proteins which fall into three distinct groups: proteins of cadherin superfamily, armadillo proteins and plakins. Desmogleins and desmocollins are desmosomal cadherins whose cytoplasmic tails associate with plakoglobins and plakophilins (the armadillo proteins). Desmoplakin interacts with plakophilin and anchors the multiprotein complex to the intermediate filaments. The desmosomes are especially abundant in epidermis and disruption of their function leads to epithelial blistering and fragility as observed in many autoimmune diseases.

The main function of tight junctions is to form selective permeability barriers in epithelial and endothelial tissues (Kim 1995). They prevent passive diffusion of molecules across the paracellular space but allow passing of selected molecules or cells. Tight junctions separate the apical and basolateral compartments of cells and are the basis for cell polarity. The barrier strand is formed by integral membrane proteins such as occluding, claudins and tricellulin (Fanning and Anderson 2009). Cytoplasmic zonula occludens proteins, ZO-1 and ZO-2 are required for the assembly of tight junctions. ZO proteins mediate the association of tight junctions with cytoskeleton through binding of F-actin and other proteins involved in the regulation of actin dynamics.

1.2.2 Cell-ECM communication

The interactions between cells and ECM determine the tissue specific functions and influence gene expression (Nelson and Bissell 2006). The majority of differentiated cells retain certain degree of plasticity to be able to respond to environmental signals and as a result, they may even change their phenotype. The response of a cell to growth factor signals can be regulated by the surrounding ECM. For example, collaboration between collagen I and platelet derived growth factor –BB regulates human dermal fibroblast migration (Li et al. 2004). Further, hepatocyte growth factor induces tube formation of kidney epithelial cells only inside the collagen matrix. The importance of cell-ECM communication in directing tissue specificity is emphasized by the fact that many invertebrates have homologs of ECM proteins and integrins.

1.2.2.1 Integrins

Integrins are the major ECM receptors. They are heterodimeric transmembrane receptors consisting of α and β subunits (Hynes 2002). They mediate cell-matrix and certain cell-cell interactions and play important roles in many biological processes such as wound healing, maintenance of tissue integrity, cell growth and survival. Integrins participate also in various pathological conditions like inflammation and invasion of cancer cells. Currently 18 α and 8 β subunits are known and they can form 24 αβ heterodimers with wide but tissue specific distribution and distinct although partially overlapping ligand binding preferences (Heino 2000; Hynes 2002). A simplified illustration of the receptors grouped by their ligand specificities is presented in Fig. 1.

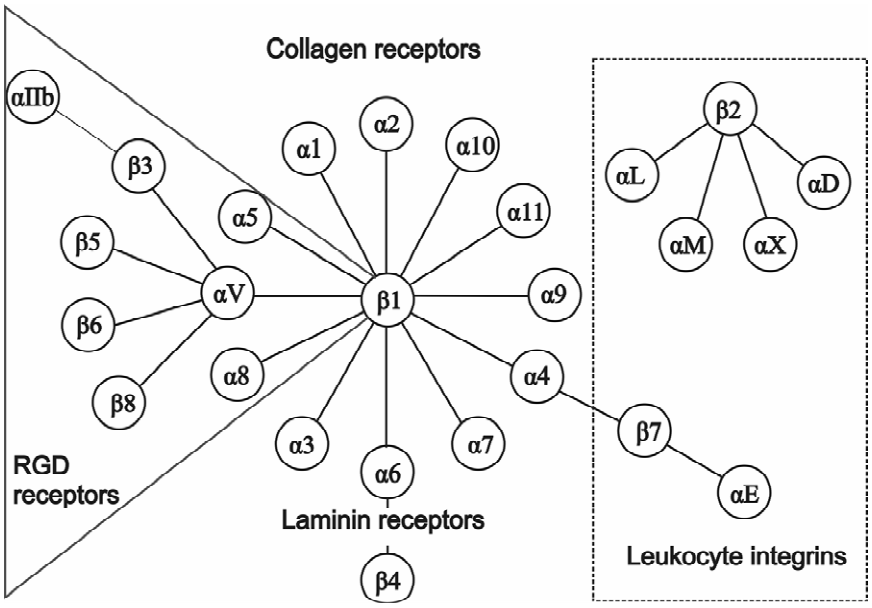

Figure 1. Integrin receptors. Adapted from (Hynes 2002).

Both α and β subunits contribute to ligand recognition. Ligands for integrin receptors are typically ECM proteins and the short intracellular tail of integrin is anchored to the actin cytoskeleton through proteins like talin, paxillin and vinculin (Zamir and Geiger 2001). Normally most integrins are present on the cell membrane in inactive, non-ligand binding state. The cytoplasmic tails of α and β subunit can interact and control the activation status. The activation can be regulated for example by growth factors and integrin ligands.

Integrin receptors lack intrinsic enzymatic activity but the cytoplasmic domains are a site of accumulation of many signaling molecules such as Src-type kinases and Src substrates (Zamir and Geiger 2001). In addition, there are proteins that interact with the transmembrane part of an integrin like adaptor protein caveolin. Interactions between cells and ECM are both dynamic and reciprocal. Through the multiple interactions integrins transmit signals from the extracellular adhesion site into the cell and vice versa. Fig. 2 illustrates the linkage between ECM and cytoskeleton provided by integrins and a number of cytosolic proteins.

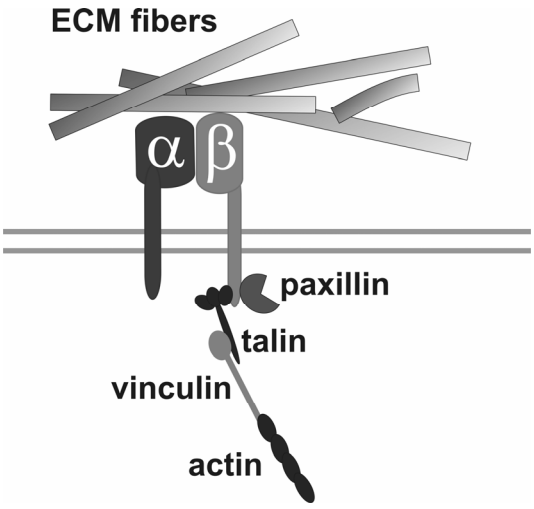

Figure 2. Integrins link the ECM to the actin cytoskeleton.

1.2.2.2 HSPGs on the cell surface

All adherent cell lines carry HSPGs on their surfaces where they modulate the recognition of a variety of ECM ligands by forming complexes with them (Bernfield et al. 1999; Park et al. 2000). The groups of syndecans and glypicans account for the majority of cell surface HSPGs. Syndecans are extended transmembrane proteins with large ectodomain and short cytoplasmic tail whereas glypicans are covalently linked to plasma membrane by glycosylphosphatidylinositol (GPI) anchor. HS chains are bound by numerous ECM proteins, proteases and their inhibitors, growth factors and their binding proteins as well as cytokines, chemokines and even some pathogens. The complex formation can result in either stimulatory or inhibitory effects often depending on the concentrations of binding

partners. Despite the diversity and abundance of ligands, the HSPG mediated interactions are not nonspecific but depend on specific HS sequences. HSPGs can also function as co-receptors for growth factors. For example, syndecan-1, the predominant HSPG on mammary epithelial cell, is a co-receptor for FGF receptor I.

1.3 Transforming growth factor β

Transforming growth factor -β (TGF-β) is a small dimeric cytokine expressed virtually in all tissues during embryonic development. It was originally found from murine sarcoma virus transformed fibroblasts as secreted activity that induced non-malignant rat kidney cells to grow in soft agar independent on anchorage to substratum (De Larco and Todaro 1978). Later, the source of the activity was identified as two distinct factors, TGF-α and -β (Roberts et al. 1982; Anzano et al. 1982) of which TGF-α belongs to the epidermal growth factor (EGF) family of growth factors. TGF-β exists as three different isoforms TGF-βs 1-3 in mammals, and isoforms found in chicken and *Xenopus* have been suggested as analogs for human TGF-β1 (Massague 1990). Despite the misleading name, TGF-βs are expressed by many normal cells and tissues rather than being a special feature of transformed cells. TGF-βs belong to a larger superfamily of structurally related cytokines which include bone morphogenic proteins (BMPs), growth differentiation factors (GDFs) and inhibins/activins (Massague 1990; Kingsley 1994). These factors have diverse effects on cell growth, morphology, differentiation and motility and are evolutionary conserved from fruit fly to human.

1.3.1 Secretion and activation of TGF-β

TGF-β1 is a prototype cytokine of the family. It is secreted as a large precursor molecule and its N-terminal propeptide is cleaved by a furin convertase during secretion (Sha et al. 1989; Dubois et al. 1995). The mature TGF-β is a dimer of 112 amino acid C-terminal subunits which are linked together via disulphide bonds. The propeptide remains non-covalently associated with the growth factor inhibiting its activity and is thus denoted latency associated peptide (LAP) (Lawrence et al. 1984; Gentry et al. 1988; Gentry and Nash 1990). This complex is called small latent complex (SL-TGF-β). In addition to conferring latency, β1-LAP is important for the folding and secretion of TGF-β1 precursor. In most cells SL-TGF-β is bound to a large glycoprotein, latent TGF-β binding protein (LTBP), through disulphide bonds between LAP and LTBP yielding a large latent complex (LL-TGF-β)(Saharinen et al. 1999). Some cells, such as glioblastomas and osteoblasts, secrete SL-TGF-βs (Olofsson et al. 1992; Dallas et al. 1994) and it can be found on the surface of platelets or activated regulatory T-cells (Grainger et al. 1995; Nakamura et al. 2004; Andersson et al. 2008). However, it has been found that LTBP facilitates the secretion and correct folding of TGF-β (Miyazono et al. 1991). It also targets the complex to the ECM forming a reservoir of the growth factor to be readily available upon appropriate stimulus (Taipale et al. 1994; Dallas et al. 1995).

Mature TGF-β needs to be released from the latent complex in order to bind its cell surface receptors and to trigger biological responses. A number of chemical, physical and biological mechanisms have been indicated in the activation of TGF-β. Exposure to extremes of pH or to urea, as well as heat treatment activates TGF-β *in vitro* (Lawrence et al. 1985; Brown et al. 1990). Proteases, such as plasmin and MMPs -2, -9, and -14 are able to activate TGF-β, some of them probably also *in vivo* (Lyons et al. 1988; Karsdal et al. 2002; Yu and Stamenkovic 2000). In addition, latent TGF-β can be activated on the surface of smooth muscle cells in co-cultures with endothelial cells, presumably by plasmin (Sato and Rifkin 1989). Other activators include thrombospondin and integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ (Schultz-Cherry and Murphy-Ullrich 1993; Schultz-Cherry et al. 1994; Munger et al. 1999; Mu et al. 2002). Contribution of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to TGF-β activity in scleroderma fibroblasts has also been implicated (Asano et al. 2005a; Asano et al. 2005b).

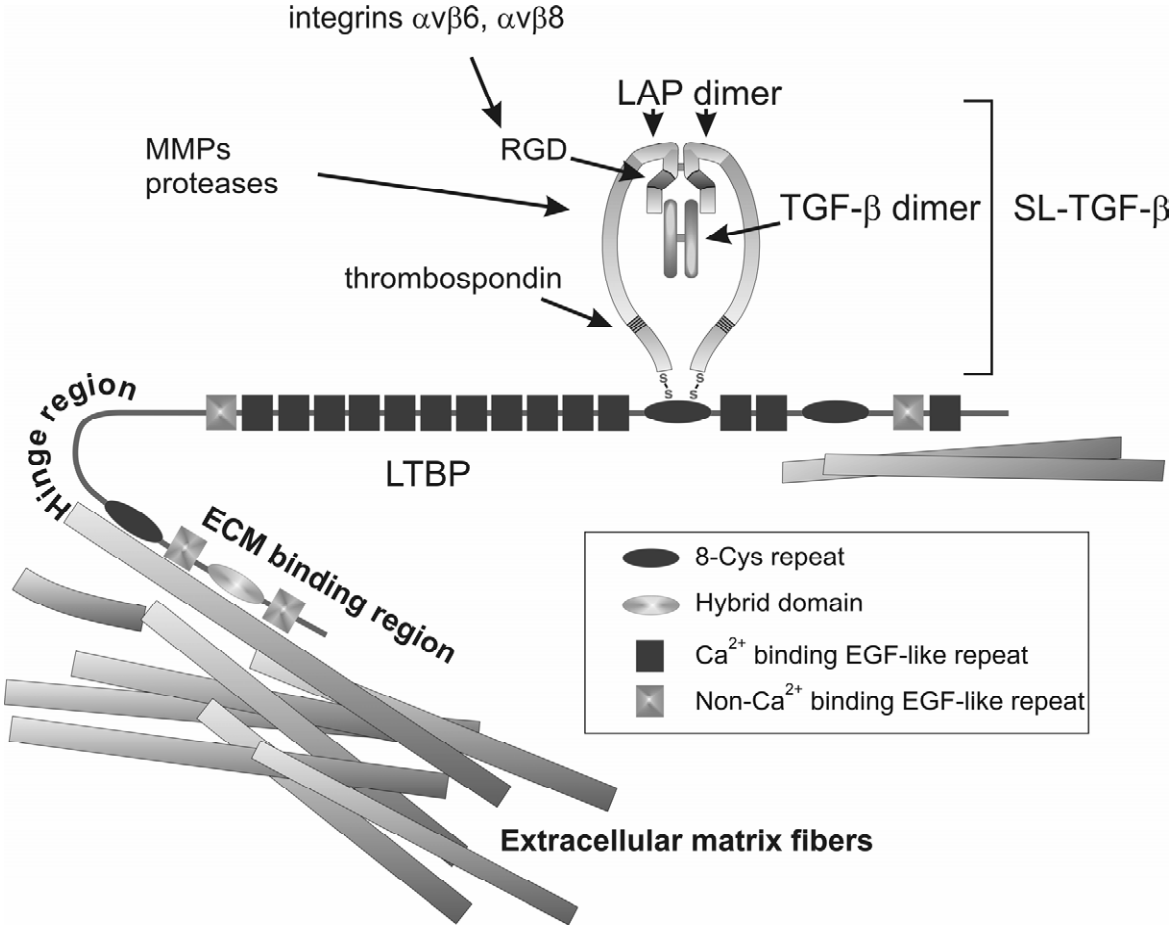

Figure 3. Illustration of the large latent TGF-β complex and activation mechanisms.
Modified from (Saharinen et al. 1999; Koli et al. 2008a).

The integrin-mediated activation of TGF-β may involve generation of intracellular and extracellular tension leading to conformational changes in LL-TGF-β as illustrated by the ability of the myofibroblast contraction to activate TGF-β1 via integrins but independently of proteases (Wipff et al. 2007). This is further supported by the requirement of FN in the $\alpha_v\beta_6$-mediated activation of TGF-β/LTBP-1 complexes (Fontana et al. 2005). By contrast, TGF-β1 activation via $\alpha_v\beta_8$ is dependent on MT1-MMP (Mu et al. 2002). Notably, the LAP of TGF-β2 lacks the RGD sequence recognized by integrins and thus cannot be activated via these mechanisms (Sheppard 2005).

Interestingly, the mice with targeted mutation in RGD sequence resulting in impaired integrin mediated TGF-β activation have phenotype which very closely resembles that of TGF-β1 null mouse (Yang et al. 2007). This would imply that the integrin mediated activation mechanisms could predominate *in vivo*. However, the proteases are important regulators of the integrin mediated activation (Mu et al. 2002; Jenkins et al. 2006). In addition, a variety of proteases including plasmin, thrombin, elastase, MMP-2 and BMP1 can liberate latent TGF-β complexes from the ECM (Taipale et al. 1992; Taipale et al. 1995; Dallas et al. 2002; Ge and Greenspan 2006). Furthermore, the expression of truncated form of LTBP-1, which is unable to associate with the ECM, leads to the formation of soluble latent TGF-β complexes and increased TGF-β activity (Mazzieri et al. 2005). Thus, the proteolytic release of latent complexes from the ECM could be the first step in the activation process.

1.3.2 TGF-β receptors and signaling

The family of cell surface receptors for TGF-β comprises subfamilies of type I and type II receptors, based on structural and functional similarities. Both types are serine/threonine transmembrane kinases and they both are essential for TGF-β signaling (Attisano and Wrana 1996; Shi and Massague 2003). Only the type II receptors bind free ligands whereas type I receptors recognize the conformationally changed ligand bound by type II receptors or the receptor-ligand interface. The formed complex is a heterotetramer which is bound to the dimeric ligand. Type II receptors phosphorylate the type I receptors on multiple serine and threonine residues. The kinase activity of type II receptor is essential for TGF-β-signaling and it is not dependent on ligand binding. Five type II receptors and seven type I receptors exist in mammals. For TGF-β ligands there is only one type II receptor (TβRII), and three distinct type I receptors, ALK1, ALK2 and ALK5/TβRI, of which TβRI mediates the majority of TGF-β responses. Other receptors or receptor combinations recognize other ligands of the TGF-β superfamily.

There are also membrane bound TGF-β binding molecules which do not have intrinsic kinase activity but contribute to TGF-β signaling as assisting receptors (Massague 1990; Massague and Chen 2000). Betaglycan is an abundantly expressed transmembrane PG also called type III receptor. It binds all TGF-β isoforms but is especially important in presenting TGF-β2 to type II receptor. A related protein endoglin is highly expressed in activated endothelial cells and is involved in vascular homeostasis. It is essential during angiogenesis as it promotes proliferation and migration of endothelial cells by regulating

the balance between ALK1 and ALK5/TβRI signaling (Lebrin et al. 2004). Some GPI-anchored TGF-β binding proteins have also been identified and suggested to regulate TGF-β responses in keratinocytes (Tam et al. 2003).

The canonical signaling pathway activated by TGF-β receptors is the Smad pathway (Massague 2000; Shi and Massague 2003), which is illustrated in Fig. 4. The type I receptors phosphorylate and thereby activate Smad2 and -3 or Smad1 and -5, depending on the receptor complex. These Smads are called receptor activated Smads (R-Smads). The access of Smad2 and -3 to TβRI can be facilitated by SARA (the Smad anchor for receptor activation) which immobilizes Smad2/3 near the cell surface. The phosphorylated R-Smads then form complexes with Co-mediator Smad, Smad4. This heteromeric complex translocates into the nucleus where it interacts with transcriptional co-activators such as CBP, p300 or ARC105 to regulate the expression of target genes. Smad6 and -7 are inhibitory Smads which can bind to the activated type I receptor and inhibit the phosphorylation of R-Smads. Smad7 can also recruit Smurf1 (Smad –ubiquitination-regulatory factor 1) or Smurf2 to the receptor complex and thus target it for degradation (Kavsak et al. 2000; Ebisawa et al. 2001). In addition, there are several proteins which can interact with Smads and affect the TGF-β response (Derynck and Zhang 2003).

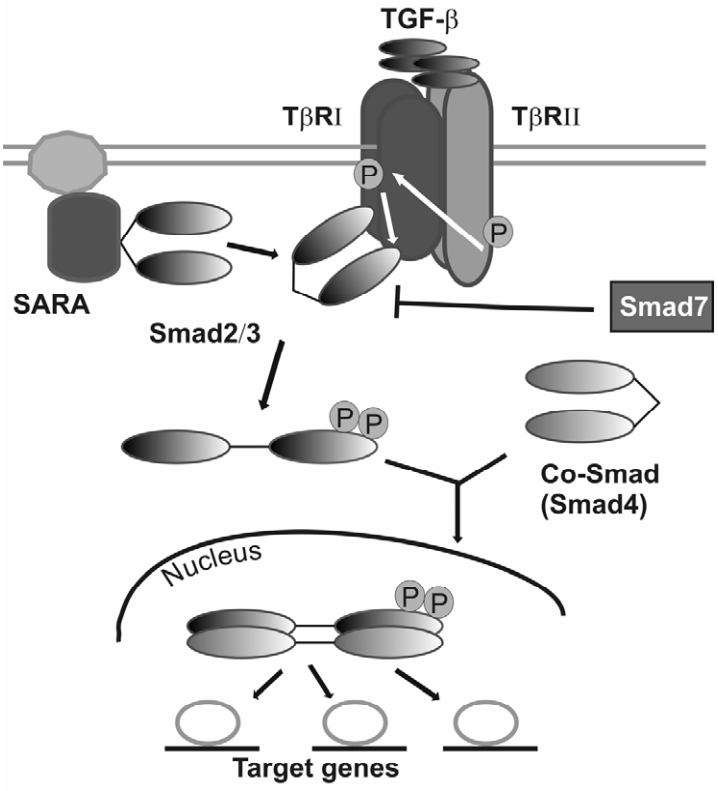

Figure 4. TGF-β signaling via Smad2/3. Modified from (Attisano and Wrana 2002) .

TGF-β can also activate other pathways including ERK, JNK and p38 mitogen activated protein kinase (MAPK) pathways (Derynck and Zhang 2003). Activation of small GTPases Rho, Rac and cdc42 and PI3 kinase has also been indicated. Interestingly, there is evidence that at least MAPK pathways converge on Smads. The crosstalk between different pathways can result in co-operation or the activities may counteract each other. For example signaling via GTPases, p38 and Smads have all been indicated during TGF-β induced epithelial-to-mesenchymal transition.

The common mechanisms for ending the Smad signaling are ubiquitination and proteasome mediated degradation of R-Smads or their dephosphorylation although the phosphatases have not been identified yet (Shi and Massague 2003). Degradation of the activated receptor complex by a proteasome also terminates TGF-β signaling.

1.3.3. TGF-βs have diverse effects on cells

TGF-β(s) exert three major functions on cells (Massague 1990; Lawrence 1996; Roberts 1996a; Letterio and Roberts 1998). Firstly, it inhibits proliferation of many cells types, typically of endodermal origin such as endothelial and epithelial cells but also some other cell types including keratinocytes. However, it can promote the growth of cells of mesenchymal origin such as fibroblasts and smooth muscle cells. The effect often depends on the growth factor concentration. Secondly, TGF-β induces the synthesis of many ECM components, such as collagen, FN, tenascin, GAGs and PGs. Moreover, it inhibits the production of many ECM degrading proteases and induces the expression of protease inhibitors, especially plasminogen activator inhibitor -1 (PAI-1) (Laiho et al. 1986). Thirdly, TGF-β is a potent suppressor of immune functions. The mechanism acts partly via the regulation of immune cell proliferation and differentiation.

The expression levels of different integrin subunits can be upregulated by TGF-β (Heino and Massague 1989). Together with increased amount of ECM components this leads to strengthened adhesion of TGF-β treated cells. However, some subunits may be strongly downregulated in certain cells. TGF-β can thus alter the repertoire of integrin receptors.

TGF-β is critically involved in wound healing, which is a process of intense accumulation and remodeling of the ECM (Massague 1990; Roberts 1996a; Toriseva and Kähäri 2009). The platelets are a rich source of TGF-β (Assoian et al. 1983) which is then available in the site of injury. It is also produced by cells of all leukocytic lineages such as monocytes and macrophages (Letterio and Roberts 1998). TGF-β is a chemoattractant for monocytes and fibroblasts and may recruit these cells to the site of wound repair (Wahl et al. 1987; Postlethwaite et al. 1987). By inducing the expression of ECM proteins TGF-β stimulates the accumulation of granulation tissue.

Vascular development is also regulated by TGF-β (ten Dijke and Arthur 2007). Low concentrations of TGF-β1 promote the proliferation and migration of endothelial cells which is required for angiogenesis, whereas high concentrations induce the production of ECM proteins typical for mature vessels (Lebrin et al. 2004). Further, TGF-β1 promotes

vascular muscularization by stimulating the differentiation of mesenchymal cells into SMCs and pericytes (ten Dijke and Arthur 2007).

TGF-βs 1-3 are highly homologous and often influence the cells in a similar manner *in vitro*. However, *in vivo,* they have distinct, although partially overlapping, spatial and temporal expression patterns and distinguishing features in their promoters indicating differential regulation. Mice with disrupted expression of TGF-β1 born as normal but die soon after weaning, at 2-3 weeks, due to massive infiltration of immune cells into various organs (Shull et al. 1992; Kulkarni et al. 1993). TGF-β2 gene targeted mice have diverse developmental defects including cranial, cardiac and lung defects (Sanford et al. 1997) and mice deficient for TGF-β3 suffer from cleft palate and abnormal lung development leading to death soon after birth (Proetzel et al. 1995). Interestingly, mice that overexpress TGF-β1 also die early (Sellheyer et al. 1993) emphasizing the fact that the effects of TGF-β are not only context but also concentration specific.

1.4 LTBP-fibrillin family

LTBP-fibrillin family of ECM proteins consists of LTBPs 1-4 (Kanzaki et al. 1990; Tsuji et al. 1990; Moren et al. 1994; Yin et al. 1995; Giltay et al. 1997; Saharinen et al. 1998) and fibrillins 1-3 (Sakai et al. 1986; Zhang et al. 1994; Nagase et al. 2001). They are often localized to long fibrillar structures which are 10-12 nm in diameter and called microfibrils (see also 1.1.4.1 Elastic fibers are assemblies of elastin and microfibrils). The molecular mass of LTBPs is 120-240 kDa whereas the fibrillins are markedly larger, ~350 kDa. They are mainly composed of cysteine rich EGF-like repeats with proline or glycine rich flanking regions. The unique feature for this protein family is the presence of eight cysteine (8-Cys) repeats that have not been detected in any other proteins. EGF-like repeats, instead, are found in many ECM and transmembrane proteins including fibulins -1 and -2, nidogen, and low density lipoprotein receptor, and they act as structural elements and mediate protein-protein interactions. LTBPs contain 15-20 and fibrillins 47 EGF-like repeats. The majority of these repeats are of calcium binding type but there are a few of non-calcium binding repeats. Four 8-Cys domains (including one hybrid domain) are present in LTBPs and nine (including two hybrid domains) in fibrillins. The basic domain structures are illustrated in Fig. 5. and reviewed in more detail in (Hyytiäinen et al. 2004).

1.4.1 LTBPs

LTBPs were first identified as binding proteins for TGF-β. LTBP-1 was isolated from platelets (Miyazono et al. 1988). It was characterized as a 125-160 kDa protein that was linked by disulfide bonds to the TGF-β1 precursor (named later as LAP) and together with non-covalently bound TGF-β1 they formed a 210 kDa latent complex. The cDNA cloning revealed that fibroblast LTBP-1 is considerably larger, 170-190 kDa, than platelet derived LTBP-1 (Kanzaki et al. 1990). LTBP-1 has been shown to exist as two N-terminally

distinct forms, LTBP-1S (the short form) and LTBP1-L (the long form) (Kanzaki et al. 1990; Tsuji et al. 1990; Olofsson et al. 1995), whose expression is driven by independent promoters (Koski et al. 1999). Other, alternatively spliced forms have also been identified (Öklü et al. 1998b; Öklü et al. 1998a; Gong et al. 1998; Michel et al. 1998). The N-terminus of LTBPs is important for the association with the ECM and LTBP-1L appeared to be more efficiently bound to the matrix (Olofsson et al. 1995; Öklü et al. 1998a). The other splice variants could protect the protein from degradation (Gong et al. 1998; Michel et al. 1998). LTBP-1 is expressed almost in all tissues but there are differences in the expression pattern of different splice variants (Tsuji et al. 1990; Olofsson et al. 1995; Öklü et al. 1998a). The expression of LTBP-1L is mainly detected in the heart, placenta, kidney and prostate whereas LTBP-1S is expressed also in the lung, spleen, stomach, ovary, testis and skeletal muscle. The cloning of mouse LTBP-1 also revealed several alternatively spliced forms with tissue specific expression patterns (Noguera et al. 2003; Weiskirchen et al. 2003). In addition, LTBP-1L is the only form expressed in mouse embryos whereas LTBP-1S predominates in adults (Weiskirchen et al. 2003).

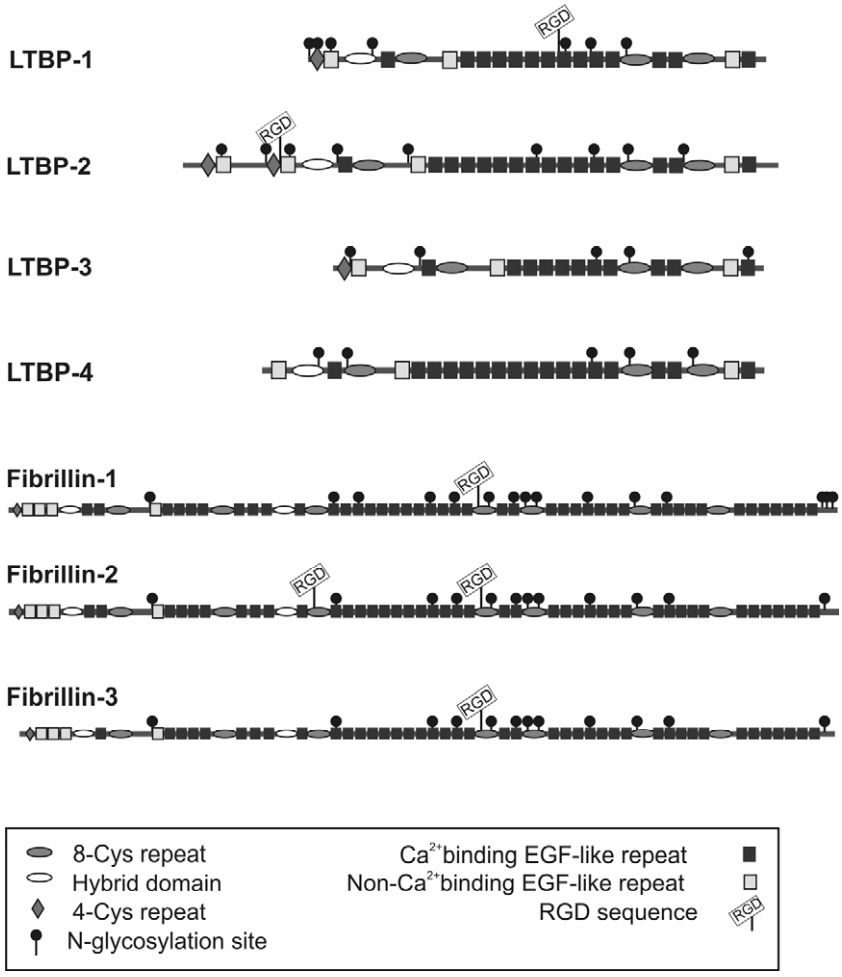

Figure 5. The domain structures of LTBPs and fibrillins. Modified from (Saharinen et al. 1999).

LTBPs -2 and -3 were identified on the basis of sequence similarity to LTBP-1 (Moren et al. 1994; Yin et al. 1995). Northern blot hybridization analysis revealed that, like LTBP-1, LTBP-2 migrates as two transcripts suggesting possible alternative splicing (Moren et al. 1994). It is highly expressed in the lung, and also in the heart, placenta, liver and skeletal muscle. The expression in the lung, dermis, perichondrium, arterial vessels, epicardium, pericardium and heart valves has been detected during rodent development (Fang et al. 1997; Shipley et al. 2000).

Human LTBP-3 exists as two different forms the longer having an additional EGF-repeat in the C-terminus (Penttinen et al. 2002). Two splice variants have been characterized for the mouse LTBP-3 as well (Yin et al. 1995). LTBP-3 is expressed especially in the skeletal muscle, heart, prostate and ovaries and to lesser extent in testis and small intestine (Penttinen et al. 2002). It is also expressed in developing bone in mouse (Yin et al. 1995).

LTBP-4 was found by searching homologues for 8-Cys repeats from sequence databases (Saharinen et al. 1998). LTBP-4 has also various alternatively spliced forms (Saharinen et al. 1998; Koli et al. 2001) of which one lacks the third 8-Cys repeat, a motif needed for the complex formation with SL-TGF-β (Koli et al. 2001). LTBP-4 is predominantly expressed in the aorta, heart, small intestine, skeletal muscle and ovaries (Giltay et al. 1997; Saharinen et al. 1998). The expression of two N-terminally distinct LTBP-4 variants, LTBP-4S (short) and -4L (long) is driven by independent promoters and gives rise to the tissue-specific expression pattern: both variants are expressed in the heart and skeletal muscle whereas LTBP-4L predominates in liver and LTBP-4S in lung as well as in small intestine (Kantola et al. 2010). Interestingly, marked differences in the secretion and processing of LTBP-4S and -4L were found. LTBP-4L was secreted in complex with TGF-β1, whereas the majority of LTBP-4S was secreted in a free form.

1.4.1.1 LTBPs -1, -3 and -4 are binding proteins for small latent TGF-β

The major role for LTBP-1 was first identified in the assembly and secretion of latent TGF-β1 as the SL-TGF-β was secreted slowly without LTBP (Miyazono et al. 1991). The majority of cells secrete TGF-β1 as large latent complexes (Olofsson et al. 1992; Flaumenhaft et al. 1993; Taipale et al. 1994; Taipale et al. 1998), which are targeted to the ECM by LTBP (Taipale et al. 1994). The binding site for SL-TGF-β in LTBPs was identified as the third 8-Cys repeat (Saharinen et al. 1996; Gleizes et al. 1996). LTBPs -1, -3 and -4 associate with SL-TGF-β whereas LTBP-2 does not (Saharinen and Keski-Oja 2000). In addition, there are differences in the complex formation properties: LTBPs -1 and -3 associate with the propeptides of all three TGF-β isoforms but LTBP-4 binds only TGF-β1-LAP and more weakly than the other LTBPs. Interestingly, LTBPs -1 and -4 are secreted also in free forms whereas LTBP-3 seems to require TGF-β for its own secretion (Chen et al. 2002; Penttinen et al. 2002).

The matrix association of LTBPs is predominantly mediated by the N-terminal domains (Saharinen et al. 1996; Unsöld et al. 2001; Chen et al. 2007; Kantola et al. 2008). Truncated large complexes can be released from the ECM by cleavage of LTBPs by

plasmin or other proteases (Taipale et al. 1994; Taipale et al. 1995). The release of latent TGF-β complex is suggested as a first step in some activation mechanisms of TGF-β (Taipale et al. 1995; Dallas et al. 2002). Antibodies against LTBP-1 inhibit the activation of TGF-β by co-cultures of endothelial and smooth muscle cells (Flaumenhaft et al. 1993), and integrin $\alpha_v\beta_6$ mediated activation requires LTBP-1 (Annes et al. 2004) suggesting a role for LTBP-1 in the activation process. LTBP-3, in addition to LTBP-1, is important for regulation of TGF-β activity during the osteogenic differentiation of mesenchymal stem cells *in vitro* (Koli et al. 2008b). In a mouse model of muscular dystrophy reduced proteolytic cleavage of LTBP-4 is associated with reduced TGF-β signaling and less severe condition (Heydemann et al. 2009). Moreover, mice, which have mutated TGF-β1 unable to form complexes with LTBPs, show decreased TGF-β signaling and suffer from inflammation and tumors (Yoshinaga et al. 2008).

Generation of gene targeted mice has revealed the special roles for different LTBP isoforms in the regulation of TGF-β: the deletion of LTBP-1L results in congenital heart defects (Todorovic et al. 2007). LTBP-3 knockout mice exhibit skeletal abnormalities such as cranio-facial defects, osteosclerosis and osteoarthritis (Dabovic et al. 2002). LTBP-4S hypomorphic mice suffer from severe pulmonary emphysema and colorectal cancer (Sterner-Kock et al. 2002). In addition, null mutations of LTBP-4 in humans result in syndrome with severe pulmonary defects and impaired gastrointestinal, musculoskeletal and dermal development (Urban et al. 2009). The effects on TGF-β activity are complex and context specific. TGF-β activity is dereased in the cardiac outflow track of mice deficient for LTBP-1L (Todorovic et al. 2007). The bone phenotype of LTBP-3 knock-out mouse is consistent with earlier studies where decreased TGF-β signaling in osteoblasts increased the trabecular bone mass (Filvaroff et al. 1999) whereas over-expression of TGF-β2 led to osteoporosis (Erlebacher and Derynck 1996). The disruption of LTBP-4S function increases TGF-β signaling in the mouse lung *in vivo* (Dabovic et al. 2009) but decreases the amount of active TGF-β secreted by the -/- fibroblasts in culture (Koli et al. 2004). In the human condition, the lack of LTBP-4 in the ECM results in increased TGF-β activity (Urban et al. 2009).

As LTBPs have only partially overlapping expression patterns and as they bind different TGF-β isoforms with different affinities it is possible that they may also differentially contribute to the TGF-β activation. At least they regulate the ECM localization of and availability of TGF-β.

LTBPs are often secreted as free forms, in excess to TGF-β implicative of other, possibly structural roles for LTBPs in the ECM (Miyazono et al. 1991; Taipale et al. 1994; Taipale et al. 1995). In addition, the expression patterns of LTBPs do not always correlate with those of TGF-βs. The existence of the LTBP-4 splice variant unable to bind SL-TGF-β (Koli et al. 2001) further implies that it could have functions not related to TGF-β. Indeed, it has recently been suggested to play a role in elastogenesis in the lung (Dabovic et al. 2009). Moreover, the predominating isoform in the lung, LTBP-4S, is mainly secreted from cells as free form (Kantola et al. 2010).

1.4.1.2 LTBP-2 – an exceptional family member

LTBP-2 is the largest member of the LTBP family. It has a domain structure similar to that of other LTBPs, especially to the long form of LTBP-1, but it also contains unique regions which make it a close relative of fibrillin-1 (Moren et al. 1994; Gibson et al. 1995; Bashir et al. 1996). The important difference to other LTBPs is that the third 8-Cys repeat of LTBP-2 lacks two amino acids between the cysteines 6 and 7 compared to corresponding repeats of the other LTBPs and can not form disulfide bonds with the LAP part of SL-TGF-β (Saharinen and Keski-Oja 2000). Biochemical characterization of LTBP-2 revealed that it is a glycosylated, calcium binding protein, which, like LTBP-1, is susceptible for proteolytic processing by plasmin and elastase (Hyytiäinen et al. 1998). Furthermore, the proteolytic cleavage resulted in the release of LTBP-2 from the ECM of fibroblasts. Interestingly, the phenotype of the knock-out mouse is strikingly more severe than the phenotypes of other *ltbp* gene targeted mice. The LTBP-2 -/- mouse embryo dies already before implantation, at E3.5-4.5, suggesting a crucial function for LTBP-2 during development (Shipley et al. 2000). The phenotype is not consistent with the mice with disrupted function of any of the TGF-β isoforms further emphasizing the function independent of TGF-β. Already earlier it was found that the bovine LTBP-2 was strongly associated with elastic fibers suggesting a structural role for it in the ECM (Gibson et al. 1995). It has also much more restricted expression pattern in embryonic mouse tissues than the other LTBPs implicative of special functions (Fang et al. 1997). A recent report has indicated human *ltbp-2* as a candidate gene for bone mineral density variation and fracture etiology (Cheung et al. 2008). In addition, the protein levels of LTBP-2 decreased during osteogenic differentiation of mesenchymal cells suggestive of involvement in bone matrix homeostasis (Koli et al. 2008b). Furthermore, a null mutation in LTBP-2 is indicated as one causative factor in primary congenital glaucoma in humans (Ali et al. 2009).

1.4.1.3 ECM associations of LTBPs

All LTBPs are targeted to the ECM after secretion (Taipale et al. 1994; Hyytiäinen et al. 1998; Koli et al. 2005). LTBP-1 colocalizes with FN and fibrillin-1 in cell cultures (Taipale et al. 1996). *In vivo*, it has been immunolocalized to microfibrillar structures in the skin, bone and heart (Raghunath et al. 1998; Dallas et al. 2000; Nakajima et al. 1997), and shown to codistribute with fibrillin-1 (Sinha et al. 2002; Isogai et al. 2003). The N-terminus of LTBP-1 was identified as the site for covalent, transglutaminase mediated cross-linking into the matrix (Nunes et al. 1997). Although the major ECM interacting sites reside in the N-terminus (Saharinen et al. 1996; Unsöld et al. 2001; Chen et al. 2007), the C-terminal motif of LTBP-1 specifically binds to N-terminus of fibrillin-1 (Isogai et al. 2003). In addition, the homologous region of fibrillin-2 also interacted with LTBP-1. However, LTBP-1 is not strongly cross-linked into microfibrils as it is not detected in microfibril extracts. The matrix assembly of LTBP-1 is dependent on FN (Dallas et al. 2005) and potentially regulated by HSPGs (Chen et al. 2007).

During the course of current studies the interaction between LTBP-2 and fibrillin-1 was discovered (Hirani et al. 2007). Accordingly, they were shown to colocalize in developing human aorta. Subsequently it was found that LTBP-2 binds fibulin-5 and regulates the assembly of elastic fibers (Hirai et al. 2007). It was suggested to act by depositing the tropoelastin preferentially on fibrillin-1 microfibrils.

LTBPs -3 and -4 associate with fibrillar structures in the fibroblast ECM but they are deposited to the matrix considerably later than LTBP-1 (Koli et al. 2005). Analogously to LTBP-1, the assembly of LTBP-4 is dependent on FN and the binding is mediated by an N-terminal domain of LTBP-4 (Kantola et al. 2008). In addition, the C-terminus of LTBP-4, but not LTBP-3, interacts with fibrillin-1 similarly to LTBP-1 and LTBP-2 (Isogai et al. 2003; Hirani et al. 2007). Notably, LTBP-4S seems to be more efficiently incorporated into the ECM than LTBP-4L in cell culture (Kantola et al. 2010).The matrix interacting domains of LTBP-3 have not been characterized so far.

1.4.2 Fibrillins

Fibrillins 1-3 (Sakai et al. 1986; Zhang et al. 1994; Nagase et al. 2001) are the main constituents of microfibrils, which are found both as associated with elastin fibers and in nonelastic tissues, such as the periodontal ligament and glomerulal mesangium of kidney (Gibson et al. 1989; Zhang et al. 1995). By electron microscopy and rotary shadowing the microfibrils have been characterized as “beads on a string” structures. The three isoforms display spatially and temporally diverse expression patterns. Fibrillin-2 is the predominating isoform during mouse embryonic development and is widely expressed by a variety of cells in mesenchyme and epithelium (Zhang et al. 1995). Its expression decreases rapidly after tissue differentiation whereas the amount of fibrillin-1 gradually increases and in the adult it is the major isoform. Exceptionally, in developing large vessels and arterioles fibrillin-1 is expressed early and the levels are higher than those of fibrillin-2. During human embryonic development fibrillins -1 and -2 display coinciding expression in most tissues such as skin, heart and lung but differ in kidney, liver, rib anlagen and notochord (Quondamatteo et al. 2002). The expression of fibrillin-3 is largely limited to fetal tissues (Corson et al. 2004). As fibrillins -1 and -2, fibrillin-3 is found in a variety of connective tissues including perichondrium, periosteum, skeletal muscle, tendon and skin. However, in contrast to the other fibrillins, it is absent from the blood vessels of lung and is neither detected in the vasculature of other organs where fibrillin-1 is abundant.

Fibrillins are involved in elastogenesis forming the template for tropoelastin deposition (Mecham 1991; Kielty et al. 2002)(see also section 1.1.4.2 Assembly of microfibrils and elastic fibers). Due to the differing expression patterns, fibrillin-2 has been suggested to regulate the early phases of elastogenesis whereas fibrillin-1 provides the elastic fibers with force-bearing structural support (Zhang et al. 1995). Special functions for fibrillin-3 have not been implicated yet. Evidence is accumulating that fibrillins are important regulators of tissue homeostasis. Several mutations have been identified in fibrillin-1 that cause a pathological human condition called the Marfan syndrome (Dietz et al. 1991;

Maslen et al. 1991). The main manifestations affect the skeletal and cardiovascular systems. Gene targeted mice with disrupted function of fibrillin-1 show similar vascular phenotype as Marfan patients but normal elastin staining of tissues (Pereira et al. 1997). The complete knock-out of fibrillin-1 leads to death soon after birth mainly due to ruptured aortic aneurysm and pulmonary defects (Carta et al. 2006). Interestingly, the pathology of Marfan syndrome is associated with dysregulation of TGF-β (Neptune et al. 2003). Moreover, a fragment of fibrillin-1 can compete with LTBP-1 for binding to N-terminus of fibrillin-1 and subsequently increase TGF-β signaling (Chaudhry et al. 2007). Hence, fibrillin-1 may regulate the bioavailability of TGF-β. Mutations in fibrillin-2 lead to a connective tissue disorder called congenital contractural arachnodactyly (Maslen et al. 1991; Putnam et al. 1995). The patients suffer from arachnodactyly, scoliosis and multiple congenital contractures. Accordingly, fibrillin-2 null mice are born with contractures of joints, which, however, resolve soon after birth (Arteaga-Solis et al. 2001). The phenotypes of the gene targeted mice suggest that fibrillins -1 and -2 perform partially overlapping functions, and the first proof of that has been obtained from aortic development (Carta et al. 2006).

The last three calcium binding EGF-like repeats in fibrillin-1 are a site for self-interaction and the C-terminal halves can assemble into multimeric globular structures corresponding to the “beads” in the beaded-string structures (Hubmacher et al. 2008). Fibrillins 1- and -2 can co-assemble and may be present in the same microfibrils but the molecular composition of fibrils depends on tissues and cell types (Charbonneau et al. 2003). The matrix assembly of fibrillin-1 is dependent on FN (Kinsey et al. 2008) and may be regulated by various interacting molecules (see section 1.1.4.2 Assembly of microfibrils and elastic fibers).

Analogously to SL-TGF-β also several BMPs, members of TGF-β superfamily, are associated with their prodomains during secretion from cells (Gregory et al. 2005; Sengle et al. 2008). These complexes are targeted to the ECM via interaction with fibrillin-1. Fibrillins may also mediate cell adhesion via domains recognized by integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_6$ (Pfaff et al. 1996; Sakamoto et al. 1996; Bax et al. 2003; Jovanovic et al. 2007).

2 Aims of the present study

When this study was initiated it had just been observed that LTBP-2 is unable to bind the SL-TGF-β (Saharinen and Keski-Oja 2000). The embryonic lethality of *ltbp-2* knockout mouse had been published (Shipley et al. 2000). The defects in the implantation of the mouse and the classical integrin recognition site, RGD, within LTBP-2 inspired us to search for putative roles for LTBP-2 in cell adhesion. In the field of cancer biology, we were interested in the roles of LTBPs in the regulation of TGF-β activity.

Specific aims of the study were:

1. To analyze whether LTBP-2 or its fragments have a role in cell adhesion.
2. To analyze the kinetics and regulation of LTBP-2 secretion and its deposition to ECM.
3. To elucidate the mechanisms by which TGF-β is released from the matrix of endothelial cells.
4. To investigate whether LTBPs play a role in the targeting and activation of TGF-β in mesothelioma.

3 Materials and methods

3.1 Cells and antibodies

Cell lines used are listed below with their American Type Culture Collection (ATCC, Manassas, VA, USA) reference number or reference.

Cell line	Description	Reference or source
CCL-137	human embryonic lung fibroblasts	ATCC; CCL-137
HT-1080	human fibrosarcoma cell line	ATCC; CCL-121
CHO	chinese hamster ovary	ATCC; CCL-61
Bowes	human melanoma cell line	ATCC; CCL-9607
G361	human melanoma cell line	ATCC; CRL-1424
WM 163	human melanoma cell line	Wistar melanoma, Wistar Institute, USA
WM 164	human melanoma cell line	Wistar melanoma, Wistar Institute, USA
WM 165	human melanoma cell line	Wistar melanoma, Wistar Institute, USA
WM 166	human melanoma cell line	Wistar melanoma, Wistar Institute, USA
WM 167	human melanoma cell line	Wistar melanoma, Wistar Institute, USA
HUVEC	human umbilical vein endothelial cells	Wistar melanoma, Wistar Institute, USA
MG-63	human osteosarcoma cell line	ATCC; CRL-1427
MEF FN+/+	mouse embryonic fibroblasts, wild type	Dr. Reinhardt Fässler, Max Planck Institute, of Biochemistry, Martinsried, Germany
MEF FN -/-	mouse embryonic fibroblasts, FN deficient	Dr. Reinhardt Fässler

Antibodies used are listed below.

Antigen	Description	Reference or source
LTBP-1	mouse monoclonal ab (MAB388)	R&D Systems, Minneapolis, MN, USA
LTBP-2, purified	rabbit polyclonal ab (abL22)	Hyytiäinen et al. 1998
β1-LAP	rabbit polyclonal ab (680)	Taipale et al. 1995
fibronectin	rabbit polyclonal ab	Sigma, St. Louis, MO, USA
fibronectin	mouse monoclonal ab (FN-15)	Sigma
collagen IV	goat polyclonal ab	Chemicon, Temecula, CA, USA
laminin	rabbit polyclonal ab	Chemicon
fibrillin-1	mouse monoclonal ab (MAB11C1)	Neomarkers, Fremont, CA, USA
fibrillin-1	rabbit polyclonal ab pAb9543	Dr. Lynn Sakai, Oregon Health and Science University, Portland, Oregon, USA
Integrin α1 subunit	mouse monoclonal ab (FB12)	Chemicon
Integrin α2 subunit	mouse monoclonal ab (1IE6)	Chemicon

Integrin α3 subunit	mouse monoclonal ab (ASC-6)	Chemicon
Integrin α4 subunit	mouse monoclonal ab (P1H4)	Chemicon
Integrin α5 subunit	mouse monoclonal ab (P1D6)	Chemicon
Integrin α6 subunit	mouse monoclonal ab (GoH3)	BD Transduction laboratories, Lexington, Ky, USA
Integrin αv subunit	mouse monoclonal ab (M9)	Chemicon
Integrin αvβ3	mouse monoclonal ab (LM609)	Chemicon
Integrin β1 subunit	mouse monoclonal ab (6S6 and P5D2)	Chemicon
paxillin	mouse monoclonal ab (177)	BD Transduction laboratories
vinculin	mouse monoclonal ab (hVIN-1)	Sigma
ERK1/2	rabbit polyclonal ab	Promega, Madison, WI, USA
P-p44/42	rabbit polyclonal ab	Cell Signaling, Beverly, MA, USA
P-Smad2	rabbit polyclonal ab (AB3849)	Millipore, Temecula, CA, USA

3.2 Production and purification of recombinant LTBP-2 and its fragments (I)

Full length recombinant LTBP-2 was produced as described (Hyytiäinen et al. 1998). Briefly, conditioned medium from CHO-L2 clone was precipitated with 30% (w/v) $(NH_4)_2SO_4$ at +4°C. The precipitate was dissolved in 50 mM Tris-HCl, pH 7.0, after which it was filtered and applied to MonoQ HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The bound proteins were eluted with 210-700 mM NaCl gradient. LTBP-2 containing fractions were combined, and 4 M urea was added to achieve the final concentration of 2 M. The sample was applied to MonoQ 5/5 column equilibrated in 50 mM Tris-HCl, pH 7.0 containing 2 M urea. Proteins were eluted with 0-1 M NaCl gradient. The fractions containing LTBP-2 were fractionated on a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.5 containing 2 M urea. The buffer of LTBP-2 fractions was then changed to phosphate buffered saline (PBS, 0.14 M NaCl, 10 mM sodium phosphate buffer, pH 7.4) with Fast Desalting column HR 10/10 (Amersham Pharmacia Biotech).

Expression and purification of LTBP-2 fragments as IgG Fc-tail fusion proteins was carried out essentially as described for LTBP-1 (Unsöld et al. 2001) and their structures are illustrated in Fig. 6.

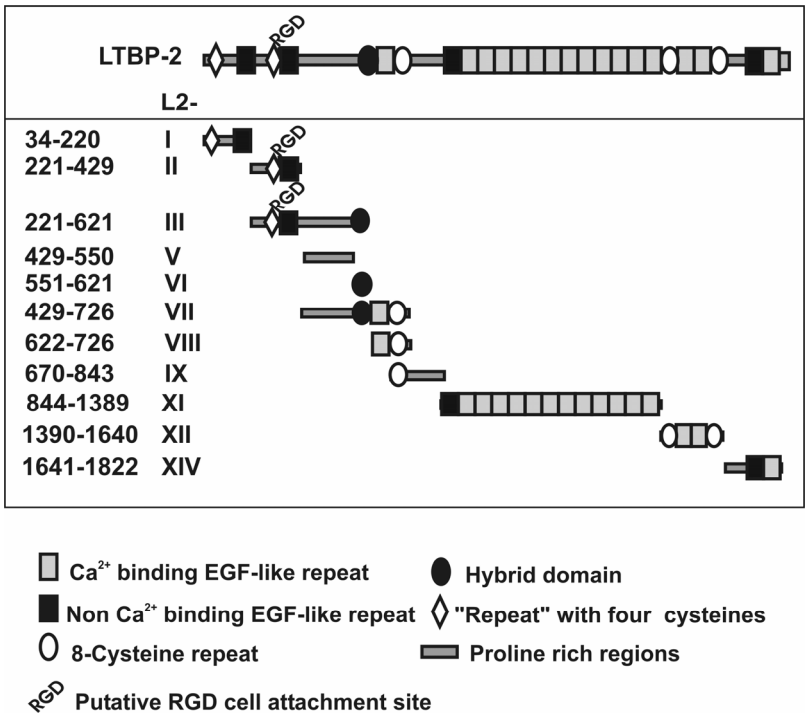

Figure 6. Schematic representation of Ig-tagged LTBP-2 fragment used for cell adhesion studies. Adopted from I.

3.3 Cell adhesion and transwell migration assays (I)

Plates of 96 wells were coated with the indicated proteins diluted in PBS at +37°C for 2 h. The wells were incubated with 1% heat denaturated bovine serum albumin (BSA) in PBS at room temperature for 30 min to prevent non-specific binding of cells. The wells were washed with PBS before the addition of cells. Cells were detached with trypsin-EDTA and suspended at density of 6.0 x 10^5 cells/ml in serum-free minimum essential medium (MEM). Cell suspension (50 µl) was then transferred to each well. In inhibition studies the cells were incubated with antibodies or other agents of interest for 15 min before transfer to the wells. The antibodies were used at concentrations up to 25 µg/ml, the RGD peptide (GRGDTP, Sigma) at 100-500 µM and heparin (Leiras, Turku, Finland) up to 800 IU/ml. Each experimental condition was tested in triplicate. The cells were allowed to attach at +37°C for 1 h, after which nonattached cells were removed by washing with PBS. Cells were then fixed and stained simultaneously with 0.1 % Coomassie Blue in 10 % acetic acid and 40 % methanol, and washed with the same fixative without the dye. Subsequently, the cells were lysed in 100 µl of 1% SDS in PBS and the absorbance was measured at 620 nm.

Transwell migration assays were performed using modified Boyden Chambers (Falcon cell culture inserts with 8 µm pores, Becton Dickinson, Franklin Lakes, NJ, USA). The upper and/or lower sides of the membranes of inserts were coated with the indicated concentrations of LTBP-2 or fibronectin (Sigma) at +37°C for 1 h, washed with PBS and

then treated with 1 % heat-inactivated BSA for 30. The inserts were then washed with PBS and placed in 24-well cell culture plates. Bowes melanoma cells were detached with trypsin-EDTA. The cells were suspended in MEM containing 0.1 % fetal calf serum (FCS) at the density of 1 x 10^6 cells/ml. Cell suspension (500 µl) was transferred to upper chambers and 750 µl of MEM with 0.1 % FCS was added to lower chambers. The cells were allowed to migrate in 5% CO_2 at +37°C for 6 h. The cells were then fixed and stained with 40 % methanol, 10 % acetic acid and 0.1 % Coomassie blue for 5 min and washed with the same fixative without the dye. The cells that had not migrated through the pores were removed from the upper surface of the membrane with cotton swabs. The number of migrated cells was counted from three randomly chosen microscope fields of each membrane. All experiments were repeated at least three times.

3.4 Protein analysis

3.4.1 Immunofluorescence stainings (I, II, III, IV)

The cells were allowed to attach to coated coverslips at +37°C for 2 h or grow on non-coated coverslips for 2-14 or 4-6 d after which the cells were fixed with 3 % paraformaldehyde (PFA) for 15 min or with methanol at -20°C for 20 min. Permeabilization of cells was performed with 0.5 % NP-40 for 5 min. The cells were then treated with 5 % BSA for 30 min, washed with PBS, and incubated with primary antibodies for 1 h. Alternatively, living cells on glass coverslips were washed with ice cold PBS and incubated with primary antibodies on ice for 20 min prior to fixing and BSA treatment. Next the coverslips were washed with PBS followed by incubation with fluorescein conjugated secondary antibodies. The coverslips were washed and mounted on glass slides using Vectashield (Vector Laboratories, Burlingame, CA). The fluorescent images were obtained using Axioplan 2 imaging microscope (Zeiss). Images were acquired with AxioCamHRc camera (Zeiss) and AxioVision3.1 software (Zeiss).

3.4.2 Isolation and plasmin digestion of ECM-associated proteins (II, III)

The cells were cultured for the indicated times and sodium deoxycholate insoluble matrices were isolated and digested with plasmin to solubilize LTBPs (Taipale et al. 1994; Koli et al. 2005). Briefly, the cell cultures were washed once with PBS and extracted by 0.5% sodium deoxycholate in 10 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl and 1% NP-40, and clarified by centrifugation at 8000 × g for 10 min. The sodium deoxycholate insoluble material was then washed once with ice-cold PBS and digested with 0.3 U/ml of plasmin (Sigma) in PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% n-octyl-β-d-glucopyranoside (Sigma) at 37°C for 2 h. Protease inhibitors (Complete®, Roche, Mannheim, Germany) were then added, and the supernatants clarified by

centrifugation. Subsequently, the soluble proteins were analyzed by SDS-PAGE and immunoblotting.

3.4.3 SDS-PAGE and immunoblotting (II, III, IV)

Proteins from the conditioned cell culture medium or ECM preparations were separated by SDS-PAGE under nonreducing conditions using 4-20% gradient Tris-HCl polyacrylamide gels (Cambrex). When necessary, conditioned cell culture medium was concentrated five-fold using Microcon YM-30 centrifugal filter devices (Millipore, Bedford, MA, USA). Sample volume was corrected for protein concentration. Electrophoretically separated proteins were transferred to nitrocellulose membranes by semi-dry blotting, and subsequently treated with 5 % non-fat milk in PBS/Triton X-100 to saturate non-specific protein-binding sites. The membranes were then reacted with antibodies, washed and detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence detection (ECL; Amersham). Incubations and washings were carried out in 50 mM Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl, 0.1 % BSA and 0.1 % Tween-20.

3.4.4 Pulse-chase and immunoprecipitation (II)

Human embryonic lung fibroblasts were cultured for 24 h or 4 days prior to labeling. First the cells were starved in methionine/cysteine –free MEM for 2 h followed by labeling with 100 µCi/ml of [^{35}S]-Cysteine (1000 Ci/mmol) in MEM containing 0.05 mM methionine at +37°C for 20 minutes. After the pulse the labeled proteins were chased in serum free MEM containing 0.05 mM methionine and 20x excess of cold L-cysteine at +37°C for 0-7 h as indicated. At each time point the chase medium was collected, cells were lysed and their ECM was extracted. After the digestion plasmin activity was inhibited with protease inhibitor cocktail, (Complete®, Roche). Next the samples were incubated with non-immune rabbit serum in an end-over-end rotary shaker at +4°C for 2 h followed by addition of protein A –sepharose (Protein A Sepharose 4B Fast Flow, Sigma) for 1 h. The beads were collected by centrifugation at 10 000 rpm for 10 minutes in a microcentrifuge. The samples were then transferred to fresh tubes and incubated with specific anti-serum against LTBP-2 (ab L22) at +4°C for 2 h followed by incubation with protein A Sepharose for 1 h. The immunocomplexes were harvested by centrifugation at 2000 rpm for 5 min. The beads were then washed twice with PBS, twice with detergent buffer (1% sodium deoxycholate, 1% TX-100, 150 mM NaCl in 50 mM Tris-HCl buffer, pH 7.0), once with high salt buffer (500 mM NaCl, 0.2% TX-100 in 20 mM Tris-HCl buffer, pH 7.0), followed by three washes with PBS. During the last wash the beads were transferred to fresh tubes. Bound proteins were eluted by heating at +95°C in SDS-PAGE sample buffer for 5 min and run in 7.5% Tris-Glycine precast gels as described above. The gels were fixed with 10% acetic acid/40% methanol for 20 min and incubated with enhancer solution (Amersham) for 30 min. Finally, the gels were dried and the signal was visualized by exposing on the film at -80°C for 1-14 days.

3.4.5 Analysis of ERK1/2 phosphorylation (III)

Cells were treated with signal pathway inhibitors for 15 min prior to treatment with PMA. After 1 h the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 % NP-40, 0.5 % NaDOC, 0.1 % SDS) containing 1 mM NaVO4, 5 mM NaF and Complete® protease inhibitor cocktail (Roche). Polypeptides from cell lysates were separated by SDS-PAGE in non-reducing conditions, and phosphorylation of ERK1/2 was analyzed using specific antibodies against phospho-ERK1/2 and total ERK1/2.

3.4.6 Gelatin zymography (III)

Polypeptides of conditioned medium were separated under nonreducing conditions using 10% polyacrylamide gels containing 1 mg/ml of gelatin. The gels were subsequently washed twice with solution containing 50 mM Tris-HCl pH 7.6, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 2.5% Triton X-100 for 15 min to remove SDS followed by a brief rinsing in washing buffer without Triton X-100. Next, the gels were incubated overnight at +37°C in a developing buffer containing 50 mM Tris-HCl pH 7.6, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100 and 0.02% $NaNO_3$. The gels were then stained with Coomassie Blue for 2 h and destained with 10% methanol, 10% acetic acid.

3.4.7 Immunohistochemistry and morphometric analysis (IV)

Paraffin-embedded tissue samples of epithelial MM were deparaffinised in xylene and dehydrated in graded alcohol. Antigens were retrieved by heating the sections in citrate buffer (pH 6.0). The sections were stained using P-Smad2 and LTBP-1 antibodies and the Novolink staining system (Novocastra, Laboratories, Newcastle upon Tyne, UK). The specificity of the staining was assessed using PBS and rabbit isotype (Zymed, San Francisco, CA, USA) negative controls. Positive immunoreactivity was visualized using DAB (Novocastra), and nuclear counterstaining by haematoxylin.

The percentage of P-Smad2 positive nuclei was calculated from each mesothelioma specimen manually using a standard hematological differential counter. Morphometric analysis of positive immunoreactivity for LTBP1-antibody was performed using Image-Pro Plus 6.1 software (Media Cybernetics, Silver Spring, MD, USA). A representative image from each section´s tumor or stroma was taken with Olympus U-CMAD3 camera (Olympus Corporation, New York, NY, USA) and QuickPHOTO CAMERA 2.1 software (Promicra, Prague, Czech Republic). The areas of positive and negative staining were calculated.

3.4.8 TGF-β activity assay (IV)

Mink lung epithelial cells stably transfected with a fragment of PAI-1 promoter fused to luciferase gene (TMLC) were provided by Dr. D.B. Rifkin (New York University School of Medicine, New York, USA). These cells produce luciferase in response to TGF-β and were used to assess the amount of active and total TGF-β in the cell conditioned medium. For measurement of total TGF-β activity the latent forms were activated by heating at +80°C for 5 min. The cells were incubated with TGF-β standards and aliquots of medium, and analyzed as described (Abe et al. 1994). The luciferase activity was normalized to cell number.

3.5 RNA isolation, reverse transcription and real-time PCR (II, III, IV)

Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA concentrations and purities were determined spectrophotometrically (BioPhotometer 6131, Eppendorf, Hamburg, Germany). Reverse transcription was carried out with Random hexamer primers (Invitrogen, Carlsbad, CA, USA) and Superscript III reverse transcriptase (Invitrogen) using 1.0 μg of total RNA according to manufacturer's instructions. The cDNAs were amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems, Foster City, CA, USA) and GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems). The results have been expressed as mRNA expression levels normalized to the levels of a gene with a constant expression (GAPDH).

3.6 Lentiviral silencing of mRNA expression (II, III)

Plasmids expressing short-hairpin-RNAs targeted against fibrillin-1 or MT1-MMP were obtained from the RNAi Consortium via Open Biosystems. Four (III) or five (IV) different constructs for each gene were tested: TRCN0000050854 (designated as construct 1=Cstr1) TRCN0000050855 (=Cstr2) TRCN0000050856 (=Cstr3) TRCN0000050857 (=Cstr4) targeted MT1-MMP. TRCN0000055893 (designated as short hairpin 1=sh1), TRCN0000055894 (=sh2), TRCN0000055895 (=sh3), TRCN0000055896 (=sh4) and TRCN0000055897 (=sh5) targeted fibrillin-1. A scrambled construct was used as a control. 293FT producer cells were cotransfected with the packaging plasmid (pCMVdr8.74; see www.tronolab.epfl.ch), envelope plasmid (pMD2-VSVG; see www.tronolab.epfl.ch) and fibrillin-1-shRNA -plasmid (hairpin-pLKO.1 vector) or control plasmid (scrambled) by using the calcium phosphate precipitation method. Normal complete culture medium (MEM) was changed to the 293FT cells 24 h after transfection. The viral supernatants were harvested for 48 h, filtered through 0.45 μm filter and transferred to human lung fibroblasts. After 16 h infection the supernatants were replaced

with complete medium for the subsequent assays. For stable shRNA expression the infected fibroblasts were subjected to selection with 5 µg/ml of puromycin for three days. The efficiency of the transduction was measured by monitoring the fibrillin-1 expression by quantitative real-time-PCR.

3.7 Patients and tissue specimens (IV)

Pleural tissue was obtained from surgical pleurectomy from patients with MM or non-malignant lung tumors (hamartomas) or from diagnostic procedures of granulomatous diseases at the Helsinki University Central Hospital. The study has been accepted by an ethical board and registered (www.hus.fi/clinicaltrials). The MM tumor array was constructed as described and the mean survival of patients after the diagnostic biopsy was taken was 18.7 months (Lindholm et al. 2009).

3.8 siRNA transfections (IV)

Human mesothelioma cells or Met5A control cells were transfected with siRNAs targeted against LTBP-1 and LTBP-3 or a negative control (Qiagen). The cells were allowed to attach for 4 hours before an overnight transfection, which was performed using Lipofectamine 2000 reagent (Invitrogen). Fresh culture medium was then changed and the cells were cultured for the indicated times before subsequent analyses. The total RNAs were isolated at 5 d. The mRNA expression levels were analyzed by quantitative RT-PCR.

3.9 Statistical analysis (IV)

PASW software was used for statistical analysis. For comparison of mean in the tumor tissue and stroma, nonparametric Mann-Whitney test was used, and the data was presented as boxplots. For analysis of correlation between different variables, Spearman´s linear regression analysis was used. For evaluation of mortality, a Kaplan-Mayer survival curve was generated.

4 Results

4.1 LTBP-2 is an adhesion protein for melanoma cells (I)

4.1.1 Melanoma cells attach to LTBP-2

To explore the effects of LTBP-2 on cell adhesion we produced recombinant LTBP-2 and partially overlapping fragments that covered the whole protein. We tested several cell lines of different origin, such as human lung fibroblasts, endothelial cells, fibrosarcoma and osteosarcoma cells, to analyze their abilities to bind to purified LTBP-2. We found that all the melanoma cell lines tested adhered to LTBP-2 (Fig. 1A). Further characterization of Bowes cells revealed that the cells adhered to LTBP-2 in a concentration dependent manner (Fig. 1B). The adhesion was associated with cell spreading and formation of extensive filopodia (Fig. 1C).

To test the specificity of the Bowes cells adhesion to LTBP-2 we treated the LTBP-2 coated wells with polyclonal antibody, abL22, generated against recombinant LTBP-2, before the adhesion assay. The treatment only partially (by 40 %) inhibited the cell adhesion (Fig. 5C), which might be explained by the observation that abL22 does not recognize the N-terminal epitopes of LTBP-2 as well as C-terminal ones (Hyytiäinen M. et al., unpublished data). Incubation of Bowes cells with soluble LTBP-2 (5 μg/ml) before plating decreased cell adhesion by ~15 % (Fig. 5C) suggesting that cells cannot efficiently bind soluble LTBP-2.

4.1.2 Bowes cell adhesion to LTBP-2 is β_1 –integrin dependent and associated with the formation of focal adhesions

The RGD sequence, commonly recognized by integrin receptors, is located to the N-terminus of LTBP-2. This raised the hypothesis that the interaction of Bowes melanoma cells with LTBP-2 could be mediated by one or more receptors of integrin type. We utilized a number of monoclonal anti-integrin antibodies in order to inhibit cell adhesion. Bowes cell adhesion to LTBP-2 was completely blocked by the anti-β_1 integrin antibody (Fig. 2A). In addition, the antibody against the integrin α_3 subunit decreased cell adhesion by 50 %, while mABs recognizing the α subunits 1, 2, 4, 5, and V did not have any effect. mAB against the integrin α_6 subunit had a small decreasing effect on the adhesion and enhanced the inhibitory effect of the anti-α_3 antibody. $\alpha_3\beta_1$ and $\alpha_6\beta_1$ are known as laminin receptors (van der Flier and Sonnenberg 2001). When used in combination, but not individually, the anti-α_3 and anti-α_6 antibodies decreased Bowes cell adhesion to LTBP-2 as efficiently as Bowes cell adhesion to laminin (Fig. 2B).

The localization of β_1 and α_3 integrins in melanoma cells adhered to LTBP-2 was analyzed by immunofluorescence. The fluorescence signal for the both of them was

localized mostly to the cell membrane or just beneath it, especially in the lamellipodia, in plaque like structures (Fig. 3). In addition, paxillin and vinculin, which are involved in connecting the integrins to the actin cytoskeleton (Zamir and Geiger 2001), were localized to the cell periphery in similar structures further confirming the formation of focal contacts after ligand recognition.

4.1.3 Melanoma cell attachment to LTBP-2 is mediated by N-terminal fragment that does not contain the RGD sequence

Since the $\alpha_3\beta_1$ integrin recognizes the RGD sequence in some ligands (Plow et al. 2000; Akula et al. 2002), we tested whether an RGD peptide could interfere with Bowes melanoma cell adhesion to LTBP-2. RGD peptide [500 μM] decreased Bowes cell adhesion to LTBP-2 by 40% while it totally prevented cell adhesion to FN and vitronectin (Fig. 5A).

Considering that cell surface PGs are important modulators of cell adhesion and HSPGs can co-operate with integrins (Bernfield et al. 1999) we examined whether Bowes cell adhesion to LTBP-2 would be affected by heparin, a wide range inhibitor of cell adhesion. Pre-treatment with heparin inhibited Bowes cell adhesion to LTBP-2 in a concentration dependent manner (Fig. 5B).

To identify the adhesive sites in the LTBP-2 protein we produced recombinant fragments covering the whole protein (Fig. 6B). Bowes cells did not adhere to any of the fragments as efficiently as to the full length LTBP-2 (Fig. 6A). The fragments most potently mediating the cell attachment were located to the N-terminus. These fragments contain a common proline–rich region. The cells could not attach to the short RGD containing fragment, but adhered to adjacent, proline rich fragment indicating that Bowes cell adhesion to LTBP-2 is not dependent on the RGD sequence.

4.1.4 LTBP-2 supports migration of Bowes melanoma cells

We explored whether melanoma cell could migrate towards LTBP-2 in addition to adhering to it. We used purified LTBP-2 for coating and FN, which is haptotactic for a variety of cell lines (Postlethwaite et al. 1987), served as a control. Bowes cells migrated very poorly when LTBP-2 was coated only on the upper surface of the membrane of cell culture insert (Fig. 7). When LTBP-2 was coated on the lower surface, the cells migrated towards it. Coating of both sides with LTBP-2 further increased cell migration, the pattern being very similar to Bowes cell migration on FN. These data suggest that LTBP-2 can support haptotactic migration of melanoma cells.

4.2 Characterization of matrix association of LTBP-2 (II)

4.2.1 LTBP-2 colocalizes temporally with fibronectin in human lung fibroblast cultures

LTBP-2 is highly expressed in the lung (Shipley et al. 2000) and thus we chose to use human embryonic lung fibroblasts as a model for analyzing matrix association of LTBP-2. We followed the formation of the ECM by immunofluorescence analysis. FN formed extensive networks already on day 2, whereas LTBP-2 was detected mostly inside the cells (Fig. 1A). Fibrillar LTBP-2 structures were visible on day 4 and they colocalized with FN. Later, LTBP-2 became organized into a fibrillar network and the maximum co-distribution with FN was observed at days 7-10. After that their co-localization started to diverge.

The expression of LTBP-2 mRNA increased about 2-fold during the first 7 days and reached the highest level, ~2.5 fold increase, at two weeks of culture (Fig. 1B). Analysis of the protein levels of secreted LTBP-2 revealed that LTBP-2 was secreted into the culture medium at relatively high levels peaking on day 4 (Fig. 1C). The peak in the secretion of LTBP-2 coincided with the time of the appearance of LTBP-2 network.

4.2.2 LTBP-2 is rapidly secreted and deposited to the ECM in 5 d fibroblast cultures

According to the protein levels, LTBP-2 was secreted into the cell culture medium already at the early time points, but it was not assembled into the matrix as assessed by immunofluorescence analysis. To explore the kinetics of LTBP-2 secretion and matrix deposition, we metabolically labeled the proteins of 1- and 5-day-old fibroblast cultures, the former being a subconfluent culture and the latter fully confluent. The labeled proteins were chased for the indicated periods of time and subjected to immunoprecipitation. We found that LTBP-2 was secreted from the cells within 3 h in 1-day-old cultures and within 1 h in 5-day-old cultures (Fig. 2). The level of secreted LTBP-2 increased up to the 7 h time point in both cultures. LTBP-2 was barely detectable in the plasmin extracted matrices of subconfluent 1-day-old cultures. In contrast, LTBP-2 was deposited to the ECM in fully confluent 5-day-old cultures already within 30 min.

4.2.3 LTBP-2 fails to assemble to the extracellular matrix of FN -/- fibroblast

Earlier work from our laboratory revealed an interaction of LTBP-2 with FN (Hyytiäinen and Keski-Oja 2003). Further, FN was found to be indispensable for the matrix assembly of both LTBP-1 and LTBP-4 (Dallas et al. 2005; Kantola et al. 2008). To explore whether FN was essential also for the matrix deposition of LTBP-2, we analyzed the levels of

LTBP-2 in the FN deficient mouse embryonic fibroblasts by immunoblotting of cell conditioned medium and by immunofluorescence. Both the wild type (+/+) and FN null (-/-) cells secreted LTBP-2 into the medium but the amount of LTBP-2 was clearly lower in the medium of FN -/- cells (Fig. 3A). When exogenous LTBP-2 was added to the medium of cells for the last 3 days of culture larger amounts of soluble LTBP-2 remained in the medium of -/- than +/+ cells as assessed by immunoblotting. Accordingly, immunofluorescence analysis indicated that LTBP-2 fibers were assembled to the ECM of FN +/+ mouse fibroblasts whereas there were no detectable LTBP-2 fibers in the ECM of FN -/- cells (Fig. 3A). The deposition of fibrillar LTBP-2 was fully rescued by cellular FN and to lesser extent by plasma FN (Fig. 3B). Fibrillin-1 deposition, which appears to be dependent on FN assembly (Kinsey et al. 2008), was accordingly rescued under the same conditions.

4.2.4 LTBP-2 colocalizes with fibrillin-1

LTBP-2 binds to fibrillin-1 but not to fibrillin-2 (Hirani et al. 2007). LTBP-2 and fibrillin-1 also colocalize in human aorta. To analyze whether they might also colocalize in cultured human lung fibroblasts we cultured fibroblasts for 2-14 days, and visualized fibrillin-1 and LTBP-2 in the ECM by immunofluorescence. Both formed visible networks by 4 days the fibrillin-1 fibers being more abundant than LTBP-2 fibers suggesting that fibrillin-1 assembly precedes the deposition of LTBP-2 (Fig. 4). From then on the two proteins partially colocalized.

We examined next whether LTBP-2 colocalizes with fibrillin-1 in other types of cultured cells. LTBP-2 fibers were assembled to the ECM of MG-63 osteosarcoma cells and human endothelial cells (HUVECs), which both exhibited also fibrillin-1 network in their ECM (Fig. 5C). The two proteins also partially colocalized.

4.2.5 Matrix association of LTBP-2 depends on fibrillin-1

Given that LTBP-2 was detected in the ECM considerably later than FN but soon after the appearance of fibrillin-1 we investigated whether the assembly of LTBP-2 into the ECM depends on fibrillin-1. We used lentiviral shRNAs to suppress the expression of fibrillin-1 and achieved ~ 95% reduction of fibrillin-1 mRNA level with the most efficient shRNA construct (shRNA4; Fig. 6A). The assembly of matrix proteins into the ECM was monitored by growing the cells on coverslips for 7 days followed by fixation and immunofluorescence analysis. Extensive networks of both LTBP-2 and fibrillin-1 were detected in the ECM of control cells (Fig. 6C). As expected, there were no detectable fibrillin-1 fibers in shRNA4 expressing cells. Interestingly, the LTBP-2 network was also ablated in fibrillin-1 deficient cells. In contrast, the assembly of FN or LTBP-1 was not affected by the suppression of fibrillin-1 expression.

4.3 LTBP-1 is released from endothelial ECM via MT1-MMP (III)

4.3.1 Release of ECM-bound LTBP-1 and LL-TGF-β in association with morphological change of endothelial cells

Activation of quiescent endothelial cells by inflammatory cytokines is associated with a morphological change of the cells from cobble-shaped into migratory spindle-shaped phenotype (Romero et al. 1997; Karasek 2007). This morphological activation can be induced by IL-1β, INF-γ, TNF-α and PMA (phorbol 12-myristate 13-acetate) (Montesano et al. 1985; Bujan et al. 1999). As expected, in response to PMA, HUVECs changed their appearance from epitheloid into elongated fibroblast-like morphology (Fig. 1A). The fate of LTBP-1 during morphological activation of HUVECs was determined by immunofluorescence analysis. LTBP-1 formed a dense fibrillar network in the ECM of confluent endothelial cells (Fig. 1A). Notably, LTBP-1 network was almost completely depleted from the ECM during 48 h PMA-treatment (Fig. 1B). LTBP1 depletion coincided with efficient degradation of another main component of subendothelial matrix, collagen type IV. In addition, the staining patterns of laminin, LTBP-2 and FN showed fragmented appearance.

Results of quantitative RT-PCR analysis indicated that HUVECs express mainly TGF-β1 out of the three known TGF-β isoforms and LTBP-1 was the main isoform of the TGF-β binding LTBPs (Fig. 2A). To detect LTBP-1 and TGF-β complexes in the crosslinked ECM, the matrices were isolated and digested with plasmin. The majority of the ECM-bound LTBP-1 was detected in free form as indicated by the abundance of 125-140 fragments in immunoblots, and 240 kDa bands representing LL-TGF-β were also detected (Fig. 2B). No other LTBP complexes were detectable with β1-LAP antibodies indicating that TGF-β1 forms complexes mainly with LTBP-1 in these cells. Markedly, the ECM of PMA-treated cells was depleted of both free LTBP-1 and LL-TGF-β1 in a time-dependent manner.

To determine whether the release of LTBP-1 from ECM involved proteolytic processing of LTBP-1 and LL-TGF-β1 we analyzed the cell conditioned media of HUVECs by immunoblotting. After treatment of the cells with increasing concentrations of PMA, LTBP-1 appeared in the conditioned medium as truncated fragments indicating proteolytical cleavage (Fig. 2C). Similarly, LL-TGF-β of a 260-280 kDa was processed to a truncated ~240 kDa form. A minor band of ~100 kDa, corresponding to the size of small latent TGF-β1 complex, was also detected. In contrast, the migration pattern of highly homologous non-TGF-β binding LTBP-2 was not notably altered by PMA indicating that the processing was specific for LTBP-1.

PMA is a direct protein kinase C (PKC) agonist which can activate ERK1/2 and PI-3 kinase pathways (Ilan et al. 1998; Taylor et al. 2006). Accordingly, PKC and ERK1/2 activities were needed for PMA induced processing of LTBP-1 (Fig. 3).

4.3.2 The processing of LTBP-1 and the release of matrix-bound TGF-β are mediated by MT1-MMP

To dissect the LTBP-1 and TGF-β releasing proteases, we examined the effects of a panel of protease inhibitors on the ECM localization of LTBP-1 by immunofluorescence analysis. The synthetic wide spectrum inhibitor against the metalloproteolytic activities of MMPs and a-disintegrin-and-metalloproteinases (ADAMs) TAPI-1 and another synthetic metalloproteinase inhibitor GM6001 markedly decreased the release of LTBP-1 suggesting the involvement of metalloproteinases in the process (Fig. 4). In contrast, the serine protease inhibitors had only negligible effects.

Given the suggested roles of MMP-2 and MMP-9 in the release of LL-TGF-β and TGF-β activation (Dallas et al. 2002; Yu and Stamenkovic 2000) we analyzed their activities of in HUVECs by gelatin zymography assay. Consistent with previous reports (Lohi et al. 1996), the expression of MMP-9 was markedly upregulated by PMA (Fig. 5A). MMP-2 was expressed at a constant level and activated in response to PMA. Since MMP-2 activation can be induced by both MT-MMPs and plasmin (Monea et al. 2002), we analyzed the effects of metalloprotease and serine protease inhibitors in this assay. Only the metalloproteinase inhibitors efficiently prevented the activation of MMP-2.

Determination of mRNA levels by quantitative RT-PCR revealed that MT1-MMP was the only notably expressed MT-MMP in HUVECs (Fig. 5B). In addition, ADAMs -10 and -17 were also expressed although on much lower level. Importantly, MT1-MMP expression was markedly induced by PMA treatment whereas the expression levels of MT2-MMP and MT3-MMP as well as ADAMS -10 and -17 remained essentially unaltered (Fig. 5C).

Considering the specific up-regulation of MT1-MMP expression and MMP-2 activation we examined the effects of tissue inhibitors of metalloproteinase (TIMPs) 1-3, which display different specificities for metalloproteinases. We found that TIMP-3, the inhibitor of all MMPs and several ADAMs, efficiently prevented the proteolytic processing of LTBP-1 and LL-TGF-β1 (Fig 6). In contrast, TIMP-1, which inhibits secreted MMPs and ADAM-10, did not have any effect. TIMP-2 inhibits both soluble MMPs and MT-MMPs and it partially prevented the proteolysis of LTBP-1 and LL-TGF-β1. The suggested involvement of MT1-MMP in the process was finally tested by lentiviral shRNA mediated suppression of its expression. Indeed, the 90% knock-down of MT1-MMP mRNA levels prevented the processing and release of LTBP-1 indicating that the PMA induced release of LTBP-1 and LL-TGF-β1 from endothelial ECM is mediated by MT1-MMP (Fig. 7).

4.4 Roles of LTBPs -1 and -3 in mesothelioma (IV)

4.4.1 Mesothelioma cells show high P-Smad2 immunoreactivity compared to stromal cells

The important role of TGF-β has been implicated in the pathogenesis of malignant mesothelioma (MM) (Fitzpatrick et al. 1995; Bielefeldt-Ohmann et al. 1996). To assess the TGF-β activity in tumor biopsies we used phosphorylated Smad2 (P-Smad2) as an indicator of active TGF-β signaling. Total of 28 human MM biopsies were tested for P-Smad2 immunoreactivity. Positive staining was observed in the nuclei both in tumor tissue and stroma (Fig. 1A). The percentage of positive nuclei was calculated from each biopsy. Approximately 70% of the tumor cell nuclei were positive for P-Smad2, whereas the mean percentage in the stroma was only ~24% (Fig. 1B). Next Kaplan-Meier plots were generated from high and low tumor P-Smad2 ratios to assess whether P-Smad2 levels in the tumor tissue would be related to patient survival. A clear trend towards better survival was observed in the samples with low P-Smad2 levels (Fig. 1C).

4.4.2 Expression levels of TGF-β and LTBP isoforms in tissue biopsies

The relative expression levels of TGF-β isoforms in mesothelioma tissue biopsies and healthy pleura were determined by quantitative RT-PCR. TGF-β1 was the most prominent isoform in both normal and tumor tissue and upregulated in all four MM samples (Fig. 2A). In addition, upregulation of TGF-β2 in MM was observed.

The analysis of mRNA expression levels of LTBP isoforms showed that all LTBPs were expressed in normal pleura but LTBP-3 was clearly the main isoform (Fig. 2B). The expression levels in MM biopsies varied markedly but the ratio of LTBP-1 to LTBP-3 mRNA level was clearly increased in 3/4 tumor samples compared to the ratio in normal pleura (Table 1).

4.4.3 LTBP-1 immunoreactivity is high in the tumor stroma

To analyze the localization of LTBP-1 protein in tissue samples we performed immunohistochemical analysis of 19 MM biopsies. Due to the lack of suitable antibodies we were unable to assess the localization of LTBP-3 or LTBP-4. We detected some LTBP-1 immunoreactivity in the tumor tissue but especially the tumor stroma was intensively stained (Fig. 2C). Quantification of LTBP-1 positive area in tumor vs. stroma by morphometric analysis indicated that ~28% of the stroma was LTBP-1 positive whereas less than 5% of the tumor area stained for LTBP-1. The correlation between LTBP-1 and P-Smad2 immunoreactivity was negative when both tumor and stroma was analysed (P=0.009, R=-0,55, Spearmans test).

4.4.4 TGF-β activity is increased in cultured mesothelioma cells

To gain insight into LTBP mediated TGF-β activation, non-tumorigenic Met5A cells and two mesothelioma cell lines (M14K and M38K) were selected for *in vitro* studies. Secreted TGF-β activity was measured from conditioned cell culture medium using the TMLC reporter cells. Elevated secretion of both active and total TGF-β was found in the medium of cultured MM cells (Fig. 3), consistent with the increased TGF-β signaling observed in tumor biopsies. Moreover, mRNA expression profiles of TGF-β and LTBP isoforms were similar to those of the tumor samples as illustrated by mRNA expression levels (Fig. 4) and increased LTBP-1 to LTBP-3 ratio in MM cells (Table 2).

4.4.5 Reduced LTBP-3 mediated targeting of TGF-β increases TGF-β activity but reduces proliferation of mesothelioma cells

To dissect the specific roles of LTBP-1 and LTBP-3 in the secretion and activation of TGF-β by MM cells we analyzed the activity of TGF-β after siRNA mediated suppression of LTBPs. Reduction of LTBP-3 but not of LTBP-1 mRNA levels resulted in increased secretion of active TGF-β by MM cells (Fig. 5A). This was not observed in Met5A cells. However, since the basal LTBP-3 mRNA expression is very high in Met5A cells, the efficiency of the siRNAs may not have been sufficient for altering the TGF-β activation. To determine the effect of the suppression of LTBP-3 expression on cell proliferation the cells were maintained in complete culture medium for 5 days after siRNA transfection followed by counting the cell numbers. In LTBP-3 siRNA transfected MM cells the increased TGF-β activity was associated with over 40% of reduction in cell number (Fig. 5B). The effect of LTBP-1 suppression on MM proliferation was only modest and the proliferation of Met5A cells was not affected.

5 Discussion

5.1 Adhesive functions of LTBP-2

LTBP-2 deficiency in the mouse leads to early embryonic lethality coinciding with the time of implantation (Shipley et al. 2000). The possible defect in implantation could suggest a role for LTBP-2 in cell adhesion. We tested several cell types, both primary and immortalized as well as cancer cells, for their ability to adhere to purified recombinant LTBP-2 coated on a cell culture plate. Unexpectedly, only melanoma cells adhered to LTBP-2. Most notably, this was applied to seven different melanoma cell lines although there was variation in the magnitude of adhesion. Further characterization of Bowes cells showed that the adhesion was mediated by β_1 integrin receptors, most likely via $\alpha_3\beta_1$ and $\alpha_6\beta_1$. The differences in the efficacy of anti-integrin antibodies could reflect the amount of the receptor on the surface of melanoma cells or the properties of the antibodies. Both types of integrin receptors are expressed in melanoma cells (Zhu et al. 2002; Tsuji et al. 2002). However, the presence or absence of these receptors on different cell types does not alone explain their ability to adhere to LTBP-2. For example, HT-1080 fibrosarcoma cells and lung fibroblasts express integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ but do not adhere to LTBP-2, which implies that additional mechanisms are involved. Various cell surface proteins or PGs, such as uPAR, thrombospondin or tetraspanins, modulate integrin functions (Ossowski and Aguirre-Ghiso 2000; Roberts 1996b). Therefore, we examined the effects of heparin in adhesion assays. Melanoma cell adhesion could be inhibited by heparin suggesting that HSPGs might be involved. However, when we explored the effect of heparan sulfate we could not observe any effect on Bowes cell adhesion to LTBP-2 (data not shown). This might implicate the involvement of very specialized form of cell surface HSPG.

We were not able to inhibit the melanoma cell adhesion to LTBP-2 with a synthetic RGD peptide even at concentration which was enough to totally prevent cell adhesion to FN and vitronectin suggestive of RGD independent mechanism. Cell adhesion to a related protein, fibrillin-1, displays cell type specific differences (Sakamoto et al. 1996). Various cell lines adhere to fibrillin-1 via RGD dependent recognition by $\alpha_v\beta_3$ integrin, and dermal fibroblasts bind also via $\alpha_5\beta_1$ (Bax et al. 2003). Chondrocytes, instead, utilize some other, non-RGD containing domain (Sakamoto et al. 1996). Moreover, $\alpha_3\beta_1$ and $\alpha_v\beta_3$ are involved in fibroblast adhesion to fibrillin-2 in RGD dependent manner. However, in fibrillins, the RGD sequence recognized by integrins is located to the fourth 8-Cys repeat where it is suggested to be part of a loop-like structure available for cellular interactions (Yuan et al. 1997; Bax et al. 2003). The RGD in LTBP-2 resides in between a domain of four cysteine repeats and an EGF-like repeat in the N-terminus of the protein. It is not a hydrophobic region but differs from the structure of the 8-Cys repeat and most likely is not as well exposed. The N-terminus of LTBP-1 and LTBP-2 mediate the ECM association (Nunes et al. 1997; Unsöld et al. 2001; Hyytiäinen and Keski-Oja 2003), which presumably affects the conformation of the protein.

We attempted to locate the adhesive site in LTBP-2 by using fragments of different lengths that covered the whole protein. However, none of the fragments was as strong adhesive substrate as the full length protein. The most potent was a proline-rich region in the N-terminus of LTBP-2 but not the adjacent fragment that contains the RGD sequence. The inefficacy of the fragments to support cell adhesion could be explained with differences in conformation compared to the full length protein, especially considering the dimeric nature of the Ig fusion proteins. However, we observed melanoma cell adhesion to the same domain produced with histidine tag but again, it was only modest compared to the adhesion to the full length LTBP-2 (Vehviläinen et al., unpublished results). This leaves us with a possibility that the integrin binding epitope is discontinuous in LTBP-2. Interestingly, the same proline-rich N-terminal domain associates with ECM and mediates anti-adhesive functions on fibroblasts adhered FN (Hyytiäinen and Keski-Oja 2003). The mechanism involves binding of LTBP or its fragment to FN and results in disruption of actin cytoskeleton of the fibroblasts. Melanoma cells and fibroblasts might recognize the corresponding fragment by different mechanisms.

Soluble LTBP-2 was not able to compete with the LTBP-2 coated on cell culture plate in adhesion assays suggesting that the adhesive properties of LTBP-2 are dependent on its deposition to substratum. It is very likely that the conformation of the protein is different in solution and potential adhesion sites are not accessible to cell surface receptors. Immobilizing the substrate to plastic or glass might resemble the *in vivo* situation.

In addition to adhesion, LTBP-2 supported Bowes melanoma cell migration. Dynamic disruption and establishment of cell-substrate contacts is important for cell migration and cancer invasion. Although cell adhesion to LTBP-2 is not a common phenomenon, the melanoma cell adherence and migration on LTBP-2 may reflect the ability of cancer cells to acquire new properties that can be beneficial during invasion and metastasizing. The melanoma cell lines used in this work were of diverse origin and their invading capacities vary. Hence, conclusions about the association of the grade of malignancy with the strength of adhesion could not be drawn. Melanoma cells are derived from melanocyte precursors and the ability of melanocytes to adhere to LTBP-2 is yet to be defined.

In the beginning of these studies there were no known molecules interacting with LTBP-2. However, during the course of this work binding of LTBP-2 to fibrillin-1 and fibulin-5 was characterized (Hirani et al. 2007; Hirai et al. 2007) but interactions with cell surface molecules have not been reported.

5.2 Association of LTBP-2 with the ECM

LTBP-2 is a microfibril-associated molecule which is highly expressed in the lung and arteries, and was recently linked to the regulation of elastic fiber assembly (Hirai et al. 2007). We wanted to examine the kinetics and determinants of matrix deposition of LTBP-2 using human lung fibroblasts as a model.

We detected LTBP-2 in the ECM of lung fibroblasts after 4 days of subculture and it partially colocalized with FN during the following days. After extended (14 d) culture these proteins started to diverge into separate fibrillar networks. This is consistent with the

observations made for the other three LTBPs (Dallas et al. 2005; Koli et al. 2005). However, clear differences were found in the timing of the LTBP-2 matrix deposition compared to the assembly of TGF-β binding LTBPs: LTBP-2 appeared in the ECM later than LTBP-1 but earlier than LTBP-3 resembling the timing of LTBP-4 assembly (Koli et al. 2005). Furthermore, there was only modest increase in the mRNA levels of LTBP-2 during 14 days of culture whereas the expression levels of LTBP-3 and -4 were induced by 7- and 4-fold suggesting that the regulation of the mRNA levels could be more important for matrix deposition of LTBP-3 and -4 than for LTBP-2. In fact, LTBP-2 was detected intracellularly and also in the conditioned medium already at early days of culture indicating that the delayed deposition of LTBP-2 due to the absence of an appropriate scaffold. Accordingly, the level of soluble LTBP-2 peaked at day 4, after which the accumulation of LTBP-2 into the ECM was detectable by both immunofluorescence and pulse-chase analysis. Consistent with studies on LTBP-1 secretion (Taipale et al. 1994; Dallas et al. 2002) LTBP-2 was rapidly secreted from cells. In confluent, 5-day-old cultures, the secretion was coupled with subsequent association of LTBP-2 to the ECM resembling the kinetics of secretion and matrix association of LTBP-1 in osteoblasts (Dallas et al. 2002). In contrast, LTBP-2 failed to efficiently assemble to the ECM of subconfluent 1-day-old cultures suggestive of requirement for an appropriate scaffold, such as FN, and implicative of incapability of LTBP-2 for self-assembly. Interestingly, LTBP-1 is detected in the ECM of fibrosarcoma cells after 2 h (Taipale et al. 1994), which is considerably later than in osteoblasts and might reflect the differences in the accumulation of ECM proteins needed for LTBP-1 assembly.

FN is widely distributed in fibrillar ECM structures, especially in provisional matrices during wound healing (Mosher 1995). It also regulates the assembly of various matrix proteins such as collagen I, thrombospondin, fibrinogen, LTBP-4 and fibrillin-1. As expected, we did not detect LTBP-2 in the ECM of FN deficient mouse embryonic fibroblasts even though exogenous LTBP-2 was added on the cells. The abundance of FN network in 1-day-old cultures of human fibroblasts and the much later deposition of LTBP-2 fibrils suggest that FN alone may not be sufficient for efficient assembly of LTBP-2.

LTBP-2 interacts with fibrillin-1 and these proteins co-localize in human embryonic aorta (Hirani et al. 2007). We found that the association of LTBP-2 with the ECM always coincided with the fibrillin-1 distribution in various cultured cell lines. In fibroblast cultures the appearance of fibrillar fibrillin-1 structures preceded the assembly of LTBP-2 network. Fibrillin-1 assembly followed similar timing in dermal fibroblasts (Kinsey et al. 2008). Thus, we hypothesized that fibrillin-1 network could promote the incorporation of LTBP-2 into the ECM. Indeed, we found that suppression of fibrillin-1 expression resulted in profound ablation of fibrillar LTBP-2 in the fibroblast ECM indicating that fibrillin-1 was necessary for efficient assembly of LTBP-2. Matrix association of fibrillin-1 depends on cellular FN and could be rescued only in part by plasma FN (Kinsey et al. 2008). Consistent with these data, we observed stronger rescue of LTBP-2 deposition to the ECM of FN -/- mouse embryonic fibroblasts by cellular than by plasma FN.

In contrast to LTBP-2, LTBP-1 is not dependent on fibrillin-1 for its matrix deposition (Dallas et al. 2005) although it binds to the same site in fibrillin-1 as LTBP-2 (Hirani et al.

2007). Our results further confirmed that fibrillin-1 knock-down does not affect the ECM association of LTBP-1. LTBP-2 thus clearly differs from LTBP-1 in the respect that its matrix assembly requires fibrillin-1. The differences in the requirements for matrix assembly may affect the tissue distribution of different LTBPs and the composition of microfibrils. A recent report described that the deposition of LTBP-1 and -4 depends also on fibrillin-1 (Ono et al. 2009). However, the fibroblasts used in that study were derived from neonatal skin of *fbn1* null mice and did not deposit fibrillin-2 fibrils. Thus, they could not rule out the possible involvement of fibrillin-2 in the incorporation of LTBP-1 into the ECM. The lung embryonic fibroblasts used in our experiments express also fibrillin-2 (Charbonneau et al. 2003). Furthermore, LTBP-1 binds to fibrillin-2 whereas LTBP-2 does not (Isogai et al. 2003; Hirani et al. 2007). Therefore, it is likely that fibrillin-2 mediates the deposition of LTBP-1 in the absence of fibrillin-1, but it cannot compensate for the loss of fibrillin-1 in the process of LTBP-2 assembly.

Deposition of fibulin-5/DANCE is dependent on fibrillin-1 in human skin fibroblasts, and LTBP-2 promotes fibulin-5 deposition on fibrillin-1 but not on fibrillin-2 and thus directs elastogenesis preferably onto fibrillin-1 microfibrils (Hirai et al. 2007). In addition, LTBP-2 knock-down rescues the fibrillin-1 independent elastin deposition. These data together with our results indicate that LTBP-2 directed inhibition of elastin deposition in the absence of fibrillin-1 results from the failure of LTBP-2 to associate with the ECM. However, the effects of LTBP-2 on elastogenesis cannot explain the early lethality of LTBP-2 -/- mice as they die already before elastin or fibrillin-1 expression is detected.

5.3 MT1-MMP mediated release of LTBP-1 and LL-TGF-β1 in endothelial cells

TGF-β plays an important role in maintaining the vascular homeostasis as well as in the regulation of angiogenesis. Since the release of ECM-bound latent TGF-β is considered as one of the regulatory steps in TGF-β activation we wanted to explore the fate of LTBP-1 during endothelial cell activation. We found that the morphological activation of endothelial cells was accompanied with proteolytic processing of ECM-bound LTBP-1 and simultaneous release of LL-TGF-β1 complexes from the ECM. This is consistent with earlier reports suggesting that the cleavage of LTBP-1 is a physiological mechanism for liberation of TGF-β from the ECM (Taipale et al. 1995; Dallas et al. 2002). The observation was cell type specific as treatment of human lung fibroblasts with PMA failed to induce processing of LTBP-1 or LL-TGF-β1. Furthermore, the soluble forms of processed LTBP-2 were not observed indicating that the phenomenon was specific for LTBP-1.

In contrast to our expectations, neither serine proteases nor secreted MMPs, which have been implicated in TGF-β activation (Dallas et al. 2002; Yu and Stamenkovic 2000), were responsible for the processing of LTBP-1. Most notably, the activation of endothelial cells was coupled with increased MT-MMP activity and strong, specific induction of the expression of MT1-MMP. In addition to synthetic wide spectrum metalloproteinase inhibitors, TIMPs -2 and -3, which inhibit secreted MMPs and MT-MMPs, prevented the

proteolytic processing of LTBP-1 and LL-TGF-β1. The involvement of MT1-MMP was finally verified by suppressing the MT1-MMP expression by ~90 % via lentivirally introduced shRNAs. As a result, the LTBP-1 network appeared intact.

The substrates of MT1-MMP include various ECM proteins such as collagen type I and IV, FN, laminin and vitronectin (Seiki 2003). It is expressed in advancing endothelial tip cells and downregulated at the time of stabilization and maturation of the vessel structure (Yana et al. 2007). In addition to degradation of ECM proteins, MT1-MMP may affect endothelial cell invasion by activating other MMPs and cleaving growth factors and their membrane-bound receptors (Seiki et al. 2003; Takino et al. 2004). Moreover, endothelial cell invasion and tube formation are inhibited by blocking MT1-MMP by TIMP-2 and TIMP-3 (Saunders et al. 2006). Our results suggest that MT1-MMP can also release ECM-bound LTBP-1 and TGF-β in conjunction with the remodeling of other subendothelial ECM proteins thus affecting pro/antiangiogenic balance. Although there is evidence that MT1-MMP can activate TGF-β (Karsdal et al. 2002), we could not confirm that in our experimental settings most likely due to too low levels of active TGF-β. This is in agreement with earlier observations which implicated the smooth muscle cells in the activation of TGF-β produced by endothelial cells (Sato et al. 1993).

5.4 LTBPs -1 and -3 may differentially regulate TGF-β activity in mesothelioma

TGF-β is an important regulator of tumor growth in mesothelioma as established by several mouse models of MM (Fitzpatrick et al. 1994; Marzo et al. 1997; Suzuki et al. 2004; Suzuki et al. 2007). In addition, TGF-β activity in pleural fluids of MM patients is markedly higher than in primary lung cancer (Maeda et al. 1994; DeLong et al. 2005). Accordingly, we found that MM tumor cells, but not the stromal cells, were strongly positive for P-Smad2 indicative of active TGF-β signaling specifically in MM tumors. Importantly, the high P-Smad2 immunoreactivity correlated with poor survival of patients. By contrast, we detected abundant immunoreactivity of LTBP-1 in the stroma of MM tumors whereas the tumor tissue stained only weakly. This is in accordance with the observed expression of LTBP-1 in the stroma of gastrointestinal carcinoma but not in cancer cells (Mizoi et al. 1993). Our results indicated that TGF-β1 was the predominant isoform expressed in MM. Considering that TGF-β1 is also a strong inducer of LTBP-1 production in normal lung fibroblasts (Weikkolainen et al. 2003) TGF-β produced by cancer cells may act in paracrine manner to stimulate the stromal cells leading to accumulation of LTBP-1. The stromal cells most likely respond differently to TGF-β than cancer cells. The reactions of cells are also dependent on the concentration of active TGF-β which does not necessarily correlate with mRNA or protein levels and which is difficult to determine in tissues. Tumor stroma plays an important role in driving cancer invasion (De Wever et al. 2008). TGF-β is able to convert normal fibroblasts of the stroma into myofibroblasts that stimulate invasion. Accumulation of LTBP-1-TGF-β complexes into the tumor stroma may provide the tumor cells a reservoir of TGF-β. On the other hand, as

LTBPs are often secreted in excess to TGF-β, LTBP-1 may alter the functions of the tumor stroma independently of TGF-β.

The mRNA levels of LTBPs in tissue biopsies varied markedly. Nevertheless, we observed that the ratio of LTBP-1 mRNA levels to LTBP-3 levels was increased in 3/4 tumor samples compared to the ratio in normal pleura. The samples were selected by an experienced pathologist and those containing more than 90% of tumor tissue were chosen for RNA analysis. However, the stromal components presumably contributed to the mRNA results.

We used established mesothelioma cell lines to dissect the roles of these LTBPs in the regulation of TGF-β. The mRNA levels of LTBP-1 were higher in MM cells than in non-malignant control cells. In contrast, LTBP-3 expression was reduced in MM cell lines, recapitulating the "switch" observed in tissue biopsies. As expected, the TGF-β activity was higher in MM cells. Suppressing the expression of LTBP-3 even more by siRNAs resulted in further increase in TGF-β activity. By contrast, downregulation of LTBP-1 expression had no effect which could have been due to insufficient efficacy of the siRNA transfections. The results suggest that LTBP-3 has an important role in targeting the LL-TGF-β to the ECM in MM cells and preventing its activation. In general, LTBPs are needed for efficient secretion of TGF-β in normal cells but osteoblasts and some cancer cells are capable of secreting small latent complexes devoid of LTBP (Bonewald et al. 1991; Olofsson et al. 1992). Moreover, defects in LTBP mediated ECM targeting can result in increased activation of TGF-β (Neptune et al. 2003; Mazzieri et al. 2005). Thus, it is possible that MM cells secrete and also activate small latent complexes when LTBP-3 levels are reduced. Alternatively, the increased TGF-β activation could take place by mechanisms specific for other LTBPs, such as LTBP-1 or -4. For example, a change in cellular LTBP expression profile from predominant LTBP-3 expression to LTBP-1 expression leads to a dramatic decrease in TGF-β activation during osteogenic differentiation of mesenchymal stem cells (Koli et al. 2008b). Our results from tumor biopsies, on the other hand, revealed a negative correlation between the LTBP-1 and P-Smad2 immunoreactivity, suggesting that the LTBP-1 mediated activation of TGF-β is not the predominant activation mechanism in either MM tumors or stroma. We were not able to assess the localization of LTBP-3 or LTBP-4 and, therefore, especially the role of LTBP-4 remains to be elucidated.

Although LTBP-3 requires association with TGF-β to be secreted from cells *in vitro*, it is possible that free LTBP-3 is deposited to the ECM *in vivo*. The free form might serve for other, possibly structural or adhesive, functions. If a shift in the ratio of LTBP-1 and LTBP-3 expression levels occurs *in vivo*, changes in their expression might, in addition to affecting TGF-β activity, alter the organization and consequently the functions of tumor stroma.

6 Perspectives

LTBP-2 is an ECM molecule with poorly known functions. The results of this study revealed that in cell culture, LTBP-2 acts as an adhesive and migration supporting substrate for melanoma cells while it is anti-adhesive for various cell types of different origin (I). LTBP-2 is expressed in the dermis of developing mice (Shipley et al. 2000) and further investigations could be directed on its expression in human dermis and especially at sites of invading melanomas. Melanoma cells are derived from melanocytes which, in turn, originate from neural crest. The expression pattern of LTBP-2 in rat brain was recently observed to be much more restricted than patterns of the other LTBPs (Dobolyi and Palkovits 2008). Thus, it would be of interest to get more insight into the role of LTBP-2 in the brain. The *in vivo* functions of LTBP-2 have remained largely unknown. Important information could be obtained by generation of a conditional knock-out mouse to overcome the implantation problems of the traditional knock-out.

The roles of LTBPs in cancers have not been extensively studied, not even in those where the role of TGF-β has been established. The results presented here suggest that LTBP-1 and LTBP-3 may differentially regulate TGF-β activity in mesothelioma (IV). Downregulation of LTBP-3 was associated with increased proliferation of MM cells in culture. Future studies should address the effects of LTBP silencing on the invasion of MM cells. In addition, LTBP-1 was found to be cleaved via MT1-MMP mediated mechanism during morphological activation of endothelial cells (III), a process which mimics angiogenesis, which, in turn, is important for tumor growth. The specific roles for different LTBP isoforms in the activation of TGF-β in cancer require further investigation.

Current data revealed the important role of fibrillin-1 in the matrix deposition of LTBP-2 (II). The association of LTBPs -1 and -4 with fibrillin-1 has also been established (Ono et al. 2009). Further studies on interactions of different LTBPs with microfibrillar proteins, such as fibrillins and fibulins, would elucidate their roles for microfibril structure and function. It has been suggested that LTBP-2 regulates elastogenesis by interacting with fibulin-5 and preferentially depositing tropoelastin on fibrillin-1 containing microfibrils (Hirai et al. 2007). All these molecules are abundantly expressed in arterial vessels and LTBP-2 is secreted by endothelial cells. Thus, it would be important to address the role of LTBP-2 in the endothelial functions such as vessel formation.

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